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INTERINDIVIDUAL VARIATION IN DRUG METABOLISM AMONG PATIENTS INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS AND PATIENTS WITH ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

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August 31, 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Doctor of Philosophy

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How HIV-related disease and medication affect the capacity to metabolize drugs was investigated in 110 HIV-positive patients including 43 with AIDS. The probe drugs acetaminophen (APAP), dextromethorphan (DM), and caffeine were used to assess the in vivo activities of glucuronidation/sulphation, cytochrome P450 (CYP) 2D6 and N-acetyltransferase 2 (NAT2). Genotypes, determined using PCR-based amplification and restriction fragment length analysis, were assigned for the latter 2 polymorphic pathways. Forty-five patients comprised the longitudinal component of the primarily cross sectional study. Urinary analyses for probe drugs and their metabolites and the compiled medical and treatment histories of the subjects were effected. The distribution of the NAT2 phenotype was unimodal and skewed toward slow acetylators as opposed to the 50:50 bimodal distribution expected. The genotype distribution was 52:48 slow:fast as expected. There were 18 discrepancies between genotype and phenotype: 12 slow acetylators with fast genotypes and 6 fast acetylators with slow genotypes. The role of disease progression in NAT2 expression was evident. Among patients whose genotype was fast the incidence of AIDS was higher in those with slow phenotypes (6/12) than those with fast phenotypes (2/14). Longitudinally, changes in phenotype from fast to slow were associated with progression of HIV-related disease. Neither APAP glucuronidation nor sulphation were affected by disease state or progression. The concomitant administration of dapsone appeared to decrease APAP-sulphation whereas zidovudine appeared to decrease APAP-glucuronidation. The data also indicated that APAP-glucuronidation may be reduced in patients who are underweight. The CYP2D6 genotype distribution between extensive (EM) and poor (PM) metabolizers was 59:2 as expected. However, 2 of the patients with an EM genotype expressed a PM phenotype and 4 others were less extensive DM metabolizers than any of the patients being treated with known CYP2D6 inhibitors. Active illness seemed to shift EM genotypes toward the PM phenotype however that alone could not explain the discrepancies. Something underlying HIV infection itself may be the cause.
RÉSUMÉ

L'objectif de cette étude était de déterminer l'effet de l'évolution du SIDA et du traitement associé sur la capacité métabolique. Cette étude a été menée chez 110 patients positifs pour le VIH dont 43 sidéens. L'acétaminophène, le dextrométhorphane, et la caféine ont été utilisés respectivement comme médicaments témoins de la glucuroconjugaision, de la sulfoconjugaision, de l'activité du CYP2D6 et de la N-acetyltransférase polymorphique (NAT2). Pour ces deux dernières voies métaboliques, les patients ont été également génotypés. De plus, parmi ces patients, 45 ont bénéficiés d'une étude longitudinale.

L'analyse des concentrations urinaires des médicaments témoins et de leurs métabolites, ainsi que l'historique clinique et thérapeutique des patients ont été effectué. Nous avons observé que l'activité métabolique NAT2-dépendente devenait unimodale avec un décalage vers les acétylateurs lents contrairement à la distribution bimodale classique. La distribution des phénotypes prédits à partir du génotype était de 52:48 (lents:rapides). Douze acétylateurs lents possédaient un génotype rapide tandis que 6 acétylateurs rapides démontraient un génotype lent. Une relation entre la progression du SIDA et l'expression phénotypique de la NAT2 était alors évidente. Ainsi, les patients ayant un génotype rapide avec un phénotype lent avaient plus de chance d'être sidéen (6 sur 12) que ceux présentant un phénotype rapide (2 sur 14). Le suivi longitudinal a confirmé le fait que la progression du SIDA était associée à un changement au niveau du phénotype de la NAT2 qui passait ainsi de rapide à lent. La distribution phénotypique de la glucuronidation ou de sulfoconjugaision ne semblaient pas être affectés par la progression du SIDA. Cependant, l'administration concomitante de dapsone chez ces patients semblait diminuer l'activité de conjugaision par sulfatation, tandis que l'administration de zidovudine était associée avec une diminution de l'activité de la glucuronidation. Il est également possible que la glucuronidation soit réduite chez les patients présentant une masse corporelle inférieure à la normale. Pour le CYP2D6, la distribution des phénotypes prédits à partir du génotype était similaire à celle observée. Cependant, deux patients avec un génotype rapide présentaient un phénotype lent. De plus, 4 patients rapides étaient aussi lents que les patients qui avaient
reçus des médicaments connus comme inhibiteurs du CYP2D6. Une maladie active semblait être associée à ce décalage phénotypique vers les métabolisateurs lents.
DEDICATION

This thesis is dedicated to my wife Brenda Smith. It is in no way an overstatement to say that without her support, encouragement, love and understanding and well, yes, badgering, this document would not have been produced. But beyond that I don't think I would have had the courage to embark upon the PhD programme if I were alone. I owe her much, no everything, as a husband and father to our children, and now as a scientist. Considering the huge familial and monetary responsibilities which she shouldered with such grace, it is only fitting that she should share in the credit for completing this degree, big time.
ACKNOWLEDGEMENTS

I am indebted to many for the completion of this research project. Financial support is gratefully acknowledged from Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR), and the Canadian Foundation for AIDS Research (CANFAR).

I would like to thank everyone in Dr. Wainer's pharmacokinetics lab and the staff of the Immune Deficiency Treatment Centre (IDTC). Of the first group I am particularly indebted to Ms. Nektaria Markoglou for her herculean efforts in completing the dextromethorphan (DM) assays, to Ms. Karen Fried for her ample good advice on myriad technical matters and to Dr. Maria Diaz-Perez, for her good example and assistance in completing the DM assays. Of the group at the IDTC, special thanks to Ms. Nadine Ayoub for her help in recruiting patients and to Drs. Gretty Deutsch, Howard Turner and Julian Falutz for their advice. Thanks also to Dr. Jean Langlais and Ms. Louise Gilbert of the flow cytometry lab for their help in processing infectious samples.

Ms. Toni Di Girolamo is in a class of her own, having been part of IDTC when the project started and of the pharmacokinetics lab by its end. She was invaluable in providing clinical expertise, helping greatly in the design of data collection forms, in recruiting patients and analyzing samples. She is among the coauthors of all three papers which comprise the body of this thesis.

The contribution of Dr. Brian Gilfix, for his genotyping work and manuscript revisions is gratefully acknowledged. I am grateful to everyone who served on my thesis committee, Professors Stan Kubow and Rhoda Blostein provided useful insights and suggestions. Additional thanks to Dr. Blostein for serving as academic advisor. Many thanks to Dr. Murray Ducharme who also provided the French translation of the abstract. Dr. Chris Tsoukas, despite his punishing schedule as director of the IDTC, was generous with his time and advice and a great help with the research.

Lastly and mostly, I am indebted to Professor Irv Wainer, first for inspiring my return to school, and also for his unstinting help and encouragement. His conception of the research plan and marshalling of the resources necessary for its completion were exemplary.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>RÉSUMÉ</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>PREFACE</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xv</td>
</tr>
<tr>
<td><strong>CHAPTER I: INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1. An overview of drug metabolism</td>
<td>2</td>
</tr>
<tr>
<td>1.1 Phase I metabolism</td>
<td>3</td>
</tr>
<tr>
<td>1.2 Phase II metabolism</td>
<td>7</td>
</tr>
<tr>
<td>1.3 Polymorphism, genotype and phenotype in drug metabolism</td>
<td>9</td>
</tr>
<tr>
<td>2. An overview of the metabolic pathways chosen for study</td>
<td>12</td>
</tr>
<tr>
<td>2.1 N-acetyltransferase 2</td>
<td>12</td>
</tr>
<tr>
<td>2.2 Cytochrome P450 2D6</td>
<td>18</td>
</tr>
<tr>
<td>2.3 Glucuronidation and Sulphation</td>
<td>20</td>
</tr>
<tr>
<td>3. HIV-infection and AIDS</td>
<td>23</td>
</tr>
<tr>
<td>3.1 Disease Characteristics</td>
<td>23</td>
</tr>
<tr>
<td>3.2 The role of nutrition in drug metabolism and HIV-infection</td>
<td>30</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>34</td>
</tr>
<tr>
<td><strong>CHAPTER II: Research Plan and Procedures</strong></td>
<td>45</td>
</tr>
</tbody>
</table>
CHAPTER III: N-Acetylation among HIV positive patients and patients with AIDS:
When is fast, fast and slow, slow? ......................................................... 49

LINKING TEXT ...................................................................................... 50
ABSTRACT ............................................................................................. 51
INTRODUCTION ...................................................................................... 52
PATIENTS AND METHODS ..................................................................... 53
  Study protocol and patients ............................................................. 53
  Phenotyping studies ...................................................................... 53
  Genotyping studies ..................................................................... 54
  Gel Electrophoresis ....................................................................... 56
  Data analysis ................................................................................. 57
RESULTS .................................................................................................. 57
  Patient characteristics ................................................................ 57
  Cross-sectional study .................................................................. 57
  Longitudinal study ..................................................................... 61
DISCUSSION ........................................................................................... 64
  Authors' note added in proof ....................................................... 68
References ............................................................................................ 68

CHAPTER IV: Glucuronidation and sulphation of paracetamol in HIV
positive patients and patients with AIDS ............................................ 71

LINKING TEXT ...................................................................................... 72
ABSTRACT ............................................................................................. 73
1. Introduction .................................................................................. 73
2. Methods ....................................................................................... 75
  2.1 Study protocol and subjects: .................................................... 75
  2.2 Metabolism studies: ............................................................... 76
  2.3 Data analysis: ....................................................................... 76
3. Results ............................................................................................ 78
  3.1 Subject characteristics: ........................................................... 78
CHAPTER V: Drug metabolism in HIV-positive patients and patients with AIDS: the genotype and expressed phenotype of the microsomal enzyme cytochrome P450 2D6
Abstract: ................................................................................................................................. A5.2

1. Introduction .......................................................................................................................... A5.3

2. Experimental ....................................................................................................................... A5.4

2.1 Chemicals .......................................................................................................................... A5.4

2.2 Standard stock solutions ................................................................................................. A5.5

2.3 Separation Chromatography ............................................................................................ A5.5

2.4 Probe-Drug Phenotyping Method .................................................................................... A5.6

2.5 Standard curves .............................................................................................................. A5.6

2.6 Assay validation .............................................................................................................. A5.7

3. Results and discussion ....................................................................................................... A5.7

3.1 Chromatography ............................................................................................................ A5.7

3.2 Validation ........................................................................................................................ A5.8

4. Conclusion ........................................................................................................................ A5.8

Acknowledgements .............................................................................................................. A5.9

REFERENCES: ....................................................................................................................... A5.9
LIST OF TABLES

CHAPTER I

Table 1. Examples of substrates, inhibitors and inducers of the major human liver cytochrome P450 enzymes involved in drug metabolism ................................. 5
Table 2. UDP-glucuronosyltransferases (UDP-GT) and their substrates .................................. 8
Table 3. Sulphotransferases (ST) and their substrates ................................................................ 9
Table 4. Substrate specificities of NAT1 and NAT2 .................................................................. 13
Table 5. Substrate specificities and inhibitors of CYP2D6 .................................................... 19
Table 6. CDC Classification Scheme of HIV Disease .............................................................. 25
Table 7. Anti-HIV medications ................................................................................................. 28
Table 8. Drugs used in managing infections commonly arising among patients with HIV infection ............................................................................................................. 29

CHAPTER III

Table I. N-Acetyltransferase (NAT2) genotypes and phenotypes of 50 HIV-positive patients and patients with AIDS ................................................................. 59
Table II. Characteristics of 12 genotypically fast patients displaying a slow phenotype ......................................................................................................................... 61
Table III. Relationship between clinical status and NAT2 phenotype among 12 patients of known genotype—longitudinal data ......................................................... 62
Table IV. Relationship between clinical status and NAT2 phenotype among 11 patients of unknown genotype—longitudinal data ....................................................... 63

CHAPTER IV

Table 1: Variability in Paracetamol Glucuronidation and Sulphation Ratios with Time of Sample Collection among Control Subjects ............................................. 79
Table 2: Variability within Control Subjects in Their Capacity to Glucuronidate and Sulphate Paracetamol Compared to That within the Control Population ........................................................................................................... 81
Table 3: Statistical Summary of Glucuronidation and Sulphation Ratios from 108 HIV Positive Patients and 36 Control Subjects.......................... 83
Table 4: Statistical Summary of Glucuronidation and Sulphation Ratios from 36 Control Subjects and 108 HIV Positive Patients Sorted According to Disease State.......................................................................................................................... 83
Table 5: Statistical Summary of Sulphation Ratios from 36 Control Subjects and 108 HIV Positive Patients Sorted According to CDC Stratum........ 83

CHAPTER V
Table 1: CYP2D6 genotypes and phenotypes of 61 HIV positive patients and patients with AIDS..................................................................................................................106
Table 2: Disease and medication characteristics of 61 HIV-positive patients sorted by [DM]/[DR] ratio..................................................................................................................109
Table 3: Odds ratios comparing pheno-subtype with A) AIDS status among all patients, B) AIDS status among all patients with an EM genotype and C) presence of active illness among all patients....................................................111

Appendix 5
Table 1 Urinary recovery of APAP and its metabolites in human urine samples (n=5) .................................................................................................................................A5.11
Table 2 Accuracy and Precision of the Analysis of APAP and its metabolites in human urine samples.................................................................................A5.12
LIST OF FIGURES

CHAPTER I

Figure 1. Principal pathways of caffeine metabolism. 17

CHAPTER III

Fig. 1. Frequency distribution of the N-acetyltransferase (NAT2) phenotype among 105 patients positive for human immunodeficiency virus (HIV). 58

CHAPTER IV

Figure 1: Variation in Spot Urine Paracetamol Molar Glucuronidation (A) and Sulphation (B) Ratios over Time. 80

Figure 2: Variation in Paracetamol Molar Glucuronidation (A) and Sulphation (B) Ratios Among 108 HIV Positive Patients (■) vs. 36 Controls (□ - Normalized Data) 82

CHAPTER V

Figure 1: Frequency distribution of CYP2D6 phenotype among 108 HIV-positive patients. 108

Figure 2: Frequency distribution of CYP2D6 phenotype among 61 HIV-positive patients of known genotype. 108

Appendix 5

Figure 1: Paracetamol and its primary metabolites: paracetamol-glucuronide and paracetamol-sulfate (conjugation of -OH group). A5.13

Figure 2: A) Chromatogram of blank urine. B) Chromatogram of drug-free urine spiked with 40 µg/ml of APAP, 4000 µg/ml of APAP-G and 1600 µg/ml of APAP-S. C) Chromatogram from a sample of a patient, 4 hours post-dosing with APAP. A5.14
PREFACE

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation." The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>137X</td>
<td>1,3,7-trimethylxanthine, caffeine</td>
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<td>1X</td>
<td>1-methylxanthine</td>
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<td>3MM</td>
<td>3-methoxymorphinan</td>
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<td>3TC</td>
<td>lamivudine</td>
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<tr>
<td>AAMU</td>
<td>5-acetylamino-6-amino-3-methyluracil</td>
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<tr>
<td>AFMU</td>
<td>5-acetylamino-6-formylamino-3-methyluracil</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APAP</td>
<td>acetyl p-aminophenol; paracetamol; acetaminophen</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AZT</td>
<td>3'-azido-3'-deoxythymidine; zidovudine</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index (M/H(^2), [mass in kilograms, height in metres])</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control &amp; Prevention</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CoASAc</td>
<td>acetyl coenzyme A</td>
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<td>CYP</td>
<td>cytochrome P450</td>
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<td>Cys</td>
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<td>dextrophan</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>extensive metabolizer</td>
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<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
</tbody>
</table>

\(\text{xv}\)
Gly  glycine
HIV  human immunodeficiency virus
HPLC  high performance liquid chromatography
IBW  ideal body weight
IDTC  Immune Deficiency Treatment Centre
INH  isoniazid
ITP  idiopathic thrombocytopenic purpura
KS  Kaposi's sarcoma
LPS  lipopolysaccharide
MAC  *mycobacterium avium* complex
MGH  Montreal General Hospital
MNL  mononuclear leukocytes
NAT1  N-acetyltransferase 1
NAT2  N-acetyltransferase 2
NHL  non-Hodgkin's lymphoma
OHL  oral hairy leukoplakia
PABA  *p*-aminobenzoic acid
PAPS  3'-phosphoadenosine-5'-phosphosulphate
PAS  *p*-aminosalicylic acid
PCP  *pneumocystis carinii* pneumonia
PCR  polymerase chain reaction
PM  poor metabolizer
PML  progressive multifocal leukoencephalopathy
SJS  Stevens-Johnson syndrome
SLE  systemic lupus erythematosus
SMX  sulphamethoxazole
ST  sulphotransferase
TBE  tris borate EDTA
TEN  toxic epidermal necrolysis

xvi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPE</td>
<td>tris phosphate EDTA</td>
</tr>
<tr>
<td>UDP-GA</td>
<td>uridine diphospho-glucuronic acid</td>
</tr>
<tr>
<td>UDP-GT</td>
<td>uridine diphospho-glucuronosyltransferase</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>vEM</td>
<td>very extensive metabolizer</td>
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<td>XO</td>
<td>xanthine oxidase</td>
</tr>
</tbody>
</table>
CHAPTER I: INTRODUCTION

Interindividual variation in drug metabolism can lead to variable pharmacokinetics and pharmacodynamics of a given medication and may originate from genetic [Eichelbaum et al. 1992; Evans et al. 1993; Vatsis et al. 1995] or environmental factors [Mucklow et al. 1980; Nash et al. 1984] or both [O'Neil et al. 1997]. Major environmental factors include the intake of concomitant medication and other xenobiotics [Baker et al. 1992; Rajaonarison et al. 1992], lifestyle factors {e.g. smoking} [Bock et al. 1987; Shenfield, 1993], the nutritional status and diet of the individual [Krishnaswamy et al. 1991; Hathcock, 1985; Lotterer et al. 1991], and the health of the individual [Tam, 1993; Esteban et al. 1997].

Therefore, patients in generally good health should not be expected to metabolize a given medication with the same efficiency as those whose illnesses are more complicated or serious. It cannot be assumed that patients taking few medications and able to maintain adequate diets will metabolize drugs in the same way as those required to take a greater number of medications or whose nutrition has become impaired. Differences in any of these factors will decrease the predictability in the outcome of drug biotransformations. Furthermore, as these factors change within an individual, so might that individual's capacity to process drugs appropriately.

In a population requiring multiple medications to treat myriad illnesses many of which have an impact on nutrition, i.e. that infected with the human immunodeficiency virus (HIV), interindividual variation in drug metabolism can be expected to be greatest and the potential for adverse effects to be highest. The work described in this thesis was undertaken for precisely these reasons.

The purpose of this research was to evaluate the impact of HIV-infection and the acquired immune deficiency syndrome (AIDS) on drug metabolism capacity. The chosen approach was to measure selected biotransformation pathways in a cohort of HIV-infected patients who, on the basis of their CD4+ cell count, would be expected to display a spectrum of illness, characteristic of advanced immunodeficiency but which would include asymptomatic patients. It was also expected that the patients would undergo disease
progression over the proposed course of the study's two years. Thus a cross sectional study with a longitudinal component was conceived. The method chosen by which to gauge the selected metabolic pathways was to administer probe drugs to the patients and subsequently measure urinary metabolite to parent ratios.

In the following pages the author will provide background from the literature to help the reader evaluate three papers which constitute the body of the thesis. This will include a brief introduction to the concepts and importance of drug metabolism with emphasis on the area of genetic polymorphism, genotype and metabolic phenotype. An overview of the metabolic pathways chosen for study and the rationale for their selection will be provided, including a review of available methods. A brief discussion of HIV-infection and its potential impact on drug metabolism and nutrition will follow. Lastly a general description of the research protocol and methods will be presented.

1. An overview of drug metabolism

Drug metabolism might be defined as that branch of pharmacology which studies what organisms do to drugs rather than what drugs do to organisms. Of course the level and extent of metabolism, what the body does to the drug, has a direct impact on the amount of drug which reaches its target and therefore, what the drug is able to do to the body. Variation in the extent of drug metabolism affects efficacy and toxicity. Depressed activity of a given pathway may lead to reduced clearance of a drug and result in increased plasma levels causing toxicity. If, however, the metabolite is the major active component, decreased efficacy could ensue from depressed metabolism.

Metabolites can be more or less active than the parent both in terms of therapeutic and toxic effects. Thus, biotransformation may include detoxification and intoxicating processes or, in terms of therapeutic impact, activating or inactivating operations. In situations where major metabolic pathways are impaired, other, minor pathways may increase in importance. Thus, toxic metabolites of little general
concern, so-called minor metabolites, may become critical to the success of therapy in certain patients.

Pharmacokinetics involve absorption, distribution, elimination and drug metabolism. Although variation in any of the first three processes will have an impact on plasma levels, it is biotransformation with which this thesis is most concerned. The methods which were chosen to evaluate specific biotransformation pathways however, are in vivo methods which comprise absorption of the probe drug, plasma protein binding and distribution to various body compartments, ultimate elimination into the urine where parent compound and metabolites can be measured, and biotransformation not only in the liver but also the gut and kidney. Thus the experiments were designed to evaluate four drug metabolism pathways within the whole patient involving auxiliary processes of pharmacokinetics.

There are various routes by which drugs are metabolized and these include the chemical reactions of oxidation, reduction, hydrolysis and hydration, among others the so-called phase I pathways, as well as the phase II reactions which include conjugation. The general function of these pathways is to facilitate the elimination of the substrate by increasing its solubility in water. Drug metabolizing enzymes evolved to catalyze reactions involving either endogenous substrates or xenobiotics of botanical origin and presumably act only so well as the drug resembles the natural compound.

1.1 Phase I metabolism

Phase I metabolism is so named because the reactions generally prepare the substrate for further pathways, usually conjugation, the so-called phase II reactions. That is not to say that phase I reactions do not often yield the most important excreted metabolites of many drugs nor that all substrates require biotransformation before they can be conjugated. Indeed isoniazid is N-acetylated (phase II) before it is hydrolysed (phase I) [Correia, 1995]. The enzymes most important in phase I metabolism comprise the microsomal mixed function oxidase system, of which the terminal oxidases are the
cytochromes P450 (CYP's).

The cytochrome P450 enzymes are membrane bound and found in the microsomal fraction (endoplasmic reticulum) of liver, kidney, lung and intestinal cells among others. These enzymes catalyze a wide variety of reactions and are responsible for the metabolism of most xenobiotics as well as many endogenous compounds (e.g. steroid hormones). CYP's constitute a superfamily of haem-containing enzymes of which some 31 isoforms have been identified to date [Nelson et al. 1993]. They are classified on the basis of their amino acid sequence similarities [Nebert, 1991] whereby isoforms of the same family (e.g. CYP's 2D6 and 2C9) are at least 40% homologous and those of the same subfamily (e.g. CYP's 2C9 and 2C19) are at least 55% homologous [Wrighton & Stevens, 1992]. The last digit(s) of the assignations were consecutively assigned as the isoforms were discovered. The CYP's are expressed in various plant and animal species and prokaryotes.

Table 1 presents details of the human CYP isoforms most important in xenobiotic biotransformations. The table is presented as a guide to the CYP's and was not meant to be exhaustive or even complete. It provides examples of substrates, inhibitors and inducers so as to facilitate the general discussion. One CYP isoform, 2D6, was studied in this research project and its characteristics will be presented in detail in later sections.

A most important enzyme not studied in this research programme is CYP3A4. Constitutively the most abundant of the CYP's [Guengerich & Shimada, 1991] it catalyzes reactions involving a host of substrates. Its induction or inhibition, by many compounds, implicate it as the source of many metabolic drug interactions. It is the most important catalyst in the metabolism of the HIV protease inhibitors ritonavir, indinavir and saquinavir as well as the non nucleoside reverse transcriptase inhibitors nevirapine [USP, 1997] and delavirdine [Cheng et al. 1997]. It is induced by nevirapine and inhibited by the protease inhibitors [USP, ibid.] and delavirdine [Cheng et al.
Table 1. Examples of substrates, inhibitors and inducers of the major human liver cytochrome P450 enzymes involved in drug metabolism

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Model substrates</th>
<th>Inhibitors</th>
<th>Inducers</th>
<th>Polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>phenacetin, caffeine, acetaminophen, acetanilide, imipramine, warfarin</td>
<td>cimetidine, furafylline</td>
<td>char broiled meat, cigarette smoke, cruciferous vegetables, omeprazole</td>
<td>no</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>coumarin, butadiene, nicotine</td>
<td>diethylidithiocarbamate, 8-methoxypsoralen</td>
<td>barbiturates</td>
<td>no</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide, ibuprofen, diclofenac, phenytoin, S-warfarin</td>
<td>sulphaphenazole, sulphipyrazole</td>
<td>rifampicin</td>
<td>yes</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin, omeprazole, citalopram, diazepam, pentamidine, imipramine</td>
<td>ketoconazole, cimetidine, omeprazole, fluoxetine, fluvoxamine</td>
<td>rifampicin, prednisone</td>
<td>yes</td>
</tr>
<tr>
<td>CYP2D6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Debrisoquine, sparteine</td>
<td>quinidine</td>
<td>none known</td>
<td>yes</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone, acetaminophen</td>
<td>disulfiram</td>
<td>isoniazid, ethanol</td>
<td>no</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Erythromycin, lidocaine, diazepam, budesonide, alfentanil, diltiazem, midazolam, nifedipine, carbamazepine, quinidine, terfenadine, imipramine, verapamil</td>
<td>ketoconazole, ciprofloxacin, grapefruit, macrolide antibiotics, cimetidine, fluvoxamine</td>
<td>rifampicin, sulphamethazine, sulphipyrazone, rifabutin, barbiturates, glucocorticoids, phenytoin, dexamethasone, carbamazepine</td>
<td>no</td>
</tr>
</tbody>
</table>

<sup>a</sup>From Birkett et al. [1993]; Flockhart [1996]; XENOtech LLC

<sup>b</sup>Please see Section 2.2 for more complete details on CYP2D6
As seen in Table 1, other drugs important in the management of HIV involve CYP3A4. Its substrates include the macrolide antibiotics, which also inhibit it as do azole antifungals and ciprofloxacin. It is induced by rifampicin, rifabutin, glucocorticoids and some sulphonamides. It is the pathway most prone to interindividual variability and perhaps most worthy of study within the HIV-infected population.

However, its exquisite sensitivity to environmental and xenobiotic forces make CYP3A4 the most difficult of isoforms to gauge in vivo. In vitro data have demonstrated that 6β-hydroxylation of cortisol [Ged et al. 1989], N-demethylation of erythromycin [Watkins et al. 1985] and N-hydroxylation of dapsone [Fleming et al. 1992] are all under the control of CYP3A4. This led to the development of in vivo probes for the enzyme. The urinary ratio of 6β-hydroxycortisol/endogenous cortisol was examined as an index by Horsman et al. [1992]. The erythromycin breath test, developed by Watkins et al. [1989], measures 14CO₂ in the breath after the intravenous administration of erythromycin, 14C-labelled at the N-methyl group, and the urinary dapsone recovery ratio [May et al. 1994] measures the N-hydroxydapsone: (N-hydroxydapsone + dapsone) ratio in the 0-8 h urine following dapsone administration. Kinirons et al. [1993] however, demonstrated an absence of correlation among these tests when applied to the same population of 30 healthy volunteers. Kivistö and Kroemer [1997] ascribe the lack of correlation to the different routes of administration and extrahepatic metabolism. They also cite Mitra et al. [1995] whose data strongly suggest CYP2E1 to be more important in dapsone hydroxylamine formation than CYP3A4.

Dextromethorphan (DM) was meant to serve as the in vivo probe for CYP3A4 (as well as CYP2D6) under the protocol designed for the research comprising this thesis (Appendix 1). Studies in human liver microsomes had indicated DM to be N-demethylated by CYP3A4 [Jacqz-Aigrain et al. 1993; Gorski et al. 1994] to 3-methoxymorphinan (3MM). The urinary ratio of
DM/3MM was therefore tested as an *in vivo* index of CYP3A4 with promising results [Ducharme *et al.* 1996; Jones *et al.* 1996]. Ducharme *et al.* [ibid.] found the ratio to reflect inhibition of the isoform by erythromycin or grapefruit juice and to be independent of CYP2D6 mediated O-demethylation, the latter confirmed by Jones *et al.* [ibid.]. In a subsequent article however, Jones *et al.* [1996a] found the test to be reliable only on the basis of a 0-72 h urine collection! Finally, Schmider *et al.* [1997] have done further *in vitro* work to demonstrate CYP2E1 to partly account for 3MM formation. The search for a reliable, safe, noninvasive *in vivo* probe for CYP3A4 must continue.

There are many other important phase I enzymes to be found in soluble fractions from liver, kidney and lung cells. They include alcohol and aldehyde dehydrogenases, xanthine and amine oxidases, aromatases, aldo-ketoreductases *etc.* The scope of this thesis precludes further discussion of those pathways.

1.2 Phase II metabolism

The phase II or conjugation reactions are classified by co-substrate and include glucuronidation, sulphation, acetylation, methylation and amino acid conjugation. The latter category includes glutathione conjugation. The reaction products are generally more hydrosoluble than the substrates, acetylation being an exception [Gibson & Skett, 1994]. The enzymes involved in these reactions are transferases (*e.g.* uridine diphospho-glucuronosyltransferase {UDPGT}, sulphotransferase, glutathione-S-transferase *etc.*). Except for the N-acetyltransferases (NAT's), phase II enzymes have not been studied to the same extent that phase I enzymes, particularly the CYP's, have.

The work presented in this thesis was concerned with glucuronidation and sulphation in general and in the activity of a specific isoform of acetyltransferase, NAT2. Although specific isoforms for the first two reactions have been isolated and/or cloned (Tables 2, 3), the level of characterization, in
terms of substrate specificity and variability is not nearly as sophisticated as for the NAT’s or CYP’s. Section 2.1 provides more information on NAT, section 2.3 covers glucuronidation and sulphation.

Table 2. UDP-glucuronosyltransferases (UDP-GT) and their substrates

<table>
<thead>
<tr>
<th>UDPGT isoform</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planar phenol (UGT1*6)</td>
<td>acetaminophen, 1-naphthol, vanillin</td>
</tr>
<tr>
<td>Complex (bulky phenol (UGT1*02)</td>
<td>propofol, carvacrol, galangin, bumentanide, ibuprofen, ketoprofen, labetolol, naproxen, propranolol, ethinyloestradiol, dapsone</td>
</tr>
<tr>
<td>Bilirubin (UGT1*1)</td>
<td>bilirubin, ethinyloestradiol, 1-naphthol, octylgallate</td>
</tr>
<tr>
<td>Oestrogen (UGT2B9)</td>
<td>clofibrate, valproate, naproxen, fenoprofen, zomepirac</td>
</tr>
<tr>
<td>Oestriol (UGT2B8)</td>
<td>oestriol, 4-nitrophenol, 1-naphthylamine</td>
</tr>
<tr>
<td>Uncloned but with distinct substrates(^b)</td>
<td>4-aminobiphenyl, amitryptiline, carbemazepine, chloramphenicol, daunomycin, digitoxigenin monodigitoxide, 5-hydroxytryptamine, imipramine, morphine, oxazepam, probenecid, rifampicin, tetracycline, temazepam, zidovudine</td>
</tr>
</tbody>
</table>

\(^a\) From Clarke & Burchell [1994]
\(^b\) Glucuronidation of each listed substrate appears to be under the control of a distinct isoform of UDPGT, as yet uncloned or of one of the following cloned isoforms for which substrate specificity has yet to be elucidated: 1*01, 1*06, 1*04, 1*4, 2B1, 2B2, 2B3, 2B4, 2B6, 2B7, 2B15

The most important of phase II pathways are those of glucuronidation, sulphation and acetylation which are covered in subsequent sections. Glutathione conjugation is an important step in facilitating the excretion of potentially toxic electrophiles. Many drugs or their phase I metabolic products are thus detoxified by glutathione-S-transferase, an enzyme
Table 3. Sulphotransferases (ST) and their substrates

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Model substrate(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermolabile phenol ST</td>
<td>dopamine, isoprenaline</td>
</tr>
<tr>
<td>Alcohol ST</td>
<td>dimetranidazole</td>
</tr>
<tr>
<td>Steroid ST</td>
<td>dihydroepiandrosterone (DHEA), oestrone</td>
</tr>
<tr>
<td>Thermostable phenol ST</td>
<td>4-nitrophenol, acetaminophen</td>
</tr>
</tbody>
</table>

* From Weinshilboum & Ottermess [1994]; Gibson & Skett [1994]

found in the cytosol of liver, kidney, gut and other tissues [Gibson & Skett, 1994].

Glutathione is a tripeptide (Gly-Cys-Glu) which, once conjugated through the Cys-S to the electrophilic substrate, may undergo further degradation to ultimately yield a cysteine conjugate. The cysteine amino group may then undergo acetylation to yield a mercapturic acid conjugate. Glutathione conjugation is considered critical in the metabolic clearance of the toxic oxidative metabolites of sulphamethoxazole (section 2.1) and acetaminophen (section 2.3).

Having mentioned the other phase II pathways in the first paragraph of this section will suffice, considering the scope of this thesis.

1.3 **Polymorphism, genotype and phenotype in drug metabolism**

Interindividual variation in metabolism arises from many factors. Genetic mutation however, gives rise to the most dramatic differences in metabolic capacity. Inactive mutant alleles have been identified for a number of xenobiotic metabolizing enzymes following observations that particular populations could be segregated into two or more defined groups which displayed widely different capacities to metabolize certain drugs.

The genotypes for many of these polymorphic enzymes have been determined and, in most cases, predict the expressed phenotype. Subjects who are homozygous for mutant alleles generally have reduced metabolic capacity and on that basis are assigned a poor metabolizer genotype. Subjects who are
hetero- or homozygous for wild type alleles generally have normal or enhanced metabolic capacity and on that basis are assigned an extensive metabolizer genotype. The use of probe drugs to gauge metabolic capacity in individuals has proven invaluable in examining the metabolic phenotypes for these polymorphic pathways [Price Evans et al. 1980; Kalow & Tang, 1993].

The NAT2 locus was one of the first found to be polymorphic. Observed differences in isoniazid metabolism combined with family studies showed slow acetylation to be an autosomal recessive trait with a distribution in healthy Caucasian populations of roughly 50:50 slow:fast acetylators [Price Evans et al. 1960]. Subsequent studies employing molecular biological techniques confirmed the existence of the NAT2 polymorphism.

CYP2D6 was found to be genetically polymorphic following the work of Mahgoub et al. [1977] who first reported the bimodal distribution of the hydroxylation of the antihypertensive debrisoquine. It had been found that a small percentage of subjects experienced an exaggerated hypotensive response due to decreased clearance of the drug via 4-hydroxylation (poor metabolizers, PM phenotype) compared to most subjects (extensive metabolizers, EM phenotype). Subsequent familial studies demonstrated the PM phenotype to be an autosomal recessive trait [Price Evans et al. 1980, Steiner et al. 1985].

There are two important components to the in vivo assessment of metabolic status of a polymorphic pathway, the genotype and expressed phenotype. There is generally a strong correlation between geno- and phenotype in healthy populations. Knowing one's genotype usually allows the accurate prediction of one's phenotype, but not always. Recent advances in allele-specific amplification and restriction fragment length polymorphism analysis have increased the ease and lessened the expense of determining genotypes to the point where it seems an attractive alternative to phenotyping. However, to know one's genotype is to know, only in qualitative terms, what the phenotype should be. It can offer no insight into where a subject might be
placed in the rather broad spectrum of activity seen within the CYP2D6 "EM" or NAT2 "fast" phenotype. In cases where non-genetic factors might perturb gene expression or enzyme activity, knowledge of the genotype alone is of little relevance.

The various methods with which to determine metabolic phenotype share the same principle: determination of a metabolic quotient following administration of a probe drug. The probe drug is given orally and the metabolic quotient is typically the molar ratio of parent to oxidized or conjugated metabolite found in the plasma or urine over a selected time period. An ideal probe drug would be one with benign metabolites excreted in the urine in easily quantified concentrations and whose production is perfectly understood. Examples of widely used metabolic probes are caffeine, for NAT2 [Grant et al. 1984; Bock et al. 1994], and dextromethorphan (DM) for CYP2D6 [Bock et al. ibid.; Jones et al. 1996].

The use of such drugs in probing polymorphic pathways within healthy populations should thus result in the assignment of an absolute phenotype (e.g. slow acetylator or extensive metabolizer via CYP2D6). However, the wide interindividual variations which can occur within a given phenotype are due only in part to interindividual genetic differences. They can also arise from environmental factors such as disease state, intake of xenobiotics, or nutritional status.

The nongenetic interindividual differences as well as changes in individual metabolic states can also be gauged with probe drugs: e.g. heavy smokers given acetaminophen were found to have a 2-fold glucuronide:parent ratio in their urine compared to moderate or non-smokers [Bock et al. 1987]. After administration of dextromethorphan, the 4-h urine sample obtained from elderly (≥65 yrs) subjects had a significantly lower N-desmethyl metabolite to parent ratio (in part indicative of CYP3A activity) than did young (≤30 yrs) subjects [Ducharme et al. 1996].
In complicated disease states such as that seen among those infected with HIV, one can expect the grey area between phenotypes to be larger than among healthy individuals with whom the probing methods were established. Thus probe drugs also serve to assess interindividual variability within various populations on an open scale. This rationale led to the design of a programme whereby patients infected with HIV, with and without AIDS, were probed with DM, caffeine and acetaminophen as a means of investigating the effects of disease state, nutritional status and concomitant medication on interindividual variation in CYP2D6, NAT2 and the glucuronidation and sulphation pathways by which acetaminophen is metabolized.

2. An overview of the metabolic pathways chosen for study

2.1 N-acetyltransferase 2

Acetylation of arylamines is under the control of two N-acetyltransferases (NAT), NAT1 and NAT2. Both enzymes require acetyl coenzyme A (CoA-S-Ac) as a cofactor. The acetate transfer occurs by a two-step, substituted-enzyme reaction mechanism [Grant et al. 1990]. NAT2 activity is largely restricted to the liver and gut whereas NAT1 is expressed more ubiquitously including in circulating blood cells [Cribb et al. 1991].

The enzymes are generally perceived as the monomorph (NAT1) and polymorphic (NAT2) forms despite recent evidence of the polymorphic attributes of NAT1 [Grant et al. 1997]. NAT2 catalyses the acetylation of mono and polycyclic aromatic amines and hydrazines such as sulphamethazine and isoniazid [Lunde et al. 1977, Grant et al. 1990]. NAT1 substrates, whose acetylation is not distributed bimodally, include p-aminobenzoic acid (PABA), and p-aminosalicylic acid (PAS) [Grant et al. ibid.] Other important substrates of NAT1 and NAT2 are presented in Table 4.
Table 4. Substrate specificities of NAT1 and NAT2

<table>
<thead>
<tr>
<th>N-acetyltransferase isoform</th>
<th>Substrates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT1</td>
<td>( p)-aminobenzoic acid (PABA)</td>
<td>1, 2</td>
</tr>
<tr>
<td></td>
<td>( p)-aminosalicylic acid (PAS)</td>
<td>1, 2</td>
</tr>
<tr>
<td></td>
<td>sulphamethazine</td>
<td>1, 2</td>
</tr>
<tr>
<td></td>
<td>sulphamethoxazole</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>isoniazid</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>hydrallazine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>dapsone</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>sulphasalazine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>procainamide</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>nitrazepam</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>phenelzine</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>caffeine</td>
<td>2</td>
</tr>
</tbody>
</table>

| NAT2                        | sulphamethazine                                  | 1, 2      |
|                             | isoniazid                                        | 1, 2      |
|                             | hydrallazine                                     | 1         |
|                             | dapsone                                          | 1         |
|                             | sulphasalazine                                   | 1         |
|                             | procainamide                                     | 1         |
|                             | nitrazepam                                       | 1         |
|                             | phenelzine                                       | 1         |
|                             | caffeine                                         | 2         |

1Lunde et al. [1977]; 2Grant et al. [1990]; 3Schröder & Price Evans [1972]; 4Cribb et al. [1993]

The polymorphism of NAT2 has recently been reviewed by Grant et al. [1997] who report 15 variant alleles to have so far been identified and polymerase chain reaction (PCR)-based genotyping tests to be available for all of them. The incidence of the slow acetylator phenotype varies with ethnicity, from 0-5% among Canadian Inuit [Armstrong & Peart, 1960; Jeanes et al. 1972], to as high as 82% among Egyptians [Hashem et al. 1969]. Less than 20% of most Asian populations are slow acetylators [Lunde et al. 1977]. The NAT2 polymorphism has also been demonstrated in other species including rabbits, mice, hamsters and rats [Zaher & Svensson, 1994].

No compounds have been found to induce increased transcription of either NAT2 or NAT1, however several studies, in animals and humans, suggest potential enhancers of NAT activity. These include immunomodulators, shown to amplify N-acetylation in rabbits and rats in vivo; precursors of CoA such as citrate, ethanol or acetate, found to increase acetylation in
man and rat hepatocytes; and hydrocortisone which causes augmented acetylation rates in rabbits in vivo [Zaher & Svensson, ibid.]. Inhibition of the enzyme can be caused by the coadministration of other NAT2 substrates [Lunde et al. 1977] and by PAS [Hanngren et al. 1970]. PAS is administered with isoniazid so as to increase the half-life of the latter drug and exercises its effect in both slow and fast acetylators.

Polymorphic drug acetylation has been implicated in the development of toxic reactions to several drugs, particularly cutaneous hypersensitivity to sulphonamides [Lunde et al. 1977]. Rashes occur in up to 5% of patients treated with sulphonamides [Rieder et al. 1991]. The most serious, and far less common, of these include a systemic lupus erythematosus (SLE)-like syndrome or even toxic epidermal necrolysis (TEN - fatal in 20-30% of cases) or its less severe form, Stevens-Johnson syndrome (SJS). Weber and Hein [1985] report studies which implicate a slow acetylator phenotype as a risk factor for hydralazine-, procainamide- and sulphasalazine-induced SLE. Wolkenstein et al. [1995] established a slow acetylator phenotype as a risk factor for sulphonamide-induced TEN or SJS. Control subjects and patients with the same severe cutaneous reactions, but to anticonvulsants, were equally distributed between slow and fast acetylator phenotypes. But 17 of 18 patients experiencing sulphonamide-induced TEN or SJS were slow acetylators. Rieder et al. [1991] reported 19 of 21 patients displaying sulphonamide hypersensitivity to be slow acetylators.

Metabolic clearance of sulphonamides is largely dependent on acetylation and therefore oxidative pathways which lead to reactive hydroxylamine derivatives would be more prominent among slow acetylators. Since slow acetylators account for roughly half the Caucasian population, the 5% incidence of adverse reactions to sulphonamides must be due to an additional defect in the detoxification pathway of those reactive metabolites, e.g. glutathione transferase deficiency [Rieder et al. ibid.].
Hypersensitivity reactions to sulphonamides occur 10 times more frequently among those infected with HIV than among the general population [Mathelier-Fusade & Leynadier, 1993]. Cutaneous hypersensitivity reactions occur in 5-30% of patients infected with HIV who receive cotrimoxazole (sulphamethoxazole-trimethoprim) as prophylaxis for pneumocystis carinii pneumonia (PCP) and up to 65% of those receiving the medication for PCP treatment [Carr et al. 1994] compared to no more than 5% in the general population [Rieder et al. 1991]. Carr et al. [ibid.] found the distribution of fast:slow acetylator phenotypes among HIV-infected patients who failed treatment with cotrimoxazole to be 1:15. The distribution seen among HIV-positive subjects who tolerated the drug and seronegative controls was 7:5 and 14:15, respectively.

Lee et al. [1993] found the distribution of the NAT2 phenotype among patients with AIDS also to be skewed. Acutely ill patients with AIDS were distributed 20:8 slow:fast. Asymptomatic HIV-positive patients, AIDS patients without acute illness, or seronegative control subjects, were equally distributed between slow:fast. The acetylator genotype among ill AIDS patients however, was found to be distributed in the same proportion as among healthy controls [Deloménie et al. 1994]. Thus patients with the slow genotype are at no greater risk of progressing to AIDS than are fast genotypes. The skew toward a slow phenotype, seen in populations whose genotype was presumed to be evenly distributed was inferred to be the result of HIV-associated processes interfering with NAT2 expression [Deloménie et al. ibid.]. A study which examined both genotype and phenotype in the same HIV-positive population [Kaufmann et al. 1996] found no observable bias in the distribution of phenotype and near-perfect agreement between genotype and phenotype.

The perplexing aspect of the association of sulphamethoxazole (SMX) hypersensitivity with slow acetylator phenotype [Carr et al. 1994; Wolkenstein et al. 1995] is that SMX is a substrate of the mostly monomorphic NAT1.
SMX acetylation does not cosegregate with the acetylation of drugs which reflect the polymorphism (e.g. isoniazid, sulphamethazine) [Cribb et al. 1993]. However, Cribb et al. [ibid.] point out that NAT2 does metabolize SMX but with lower affinity than NAT1. They suggest that variation in either NAT1 or NAT2 activity could play an important role in one's susceptibility to SMX toxicity.

The determination of acetylator phenotype has been recommended for patients who are to undergo long-term treatment with NAT2 substrates [Lunde et al. 1977]. The phenotype is determined from the metabolic quotient of an appropriate probe drug. Most of the work establishing the existence of the polymorphism used isoniazid (INH) as the probe [ibid.]. However, after acetylation INH is hydrolyzed to the hepatotoxic acetylhydrazine [Correia, 1995] and is therefore not an ideal probe. Determination of the metabolic quotient also depends on obtaining a plasma sample. Sulphamethazine replaced INH as the probe of choice through the 1970's [Lunde et al. ibid.] until caffeine was demonstrated to serve ideally in identifying slow and fast acetylators [Grant et al. 1984].

Caffeine itself is not acetylated during its metabolism (Figure 1) and so the metabolic quotient is not the usual parent/metabolite ratio. In fact the substrate is not known (represented by [?] in Figure 1). A number of ratios have been tried [Vincent-Viry et al. 1994] all incorporating 5-acetylamino-6-formylamino-3-methyluracil (AFMU) in the numerator and the sum of a number of the other terminal metabolites in the denominator. Vincent-Viry et al. [ibid.] demonstrated that no ratio was better than the simplest, AFMU/1-methylxanthine (1X).

The obvious appeal of caffeine as a probe stems from its ubiquitous use and availability, its complete lack of serious side effects and the fact that the metabolic quotient is determined in a urine sample which may be collected at any time between 2 and 6 hours after ingestion of the caffeine [Grant et al.}
1984]. It is critical, however, that samples be titrated to a pH of 3.5 or less as soon after collection as possible to prevent the spontaneous deamidation of AFMU to 5-acetylamino-6-amino-3-methyluracil (AAMU). Alternatively, some protocols add base to urine samples to quantitatively effect the deamidation [Denaro et al. 1996] and assess the AAMU/1X ratio.

Figure 1. Principal pathways of caffeine metabolism. Enzyme names appear beside arrows; parentheses indicate unconfirmed enzymes. NAT2: N-acetyltransferase 2, XO: xanthine oxidase, CYP: cytochrome P450, 137X: 1,3,7-trimethylxanthine (caffeine), [?]: unknown intermediate metabolite.

The AFMU/1X metabolic ratio correlates superbly with other indices of polymorphic acetylation. A study in which subjects undergoing liver biopsy
were also phenotyped with caffeine allowed the in vitro determination of liver cytosolic N-acetyltransferase using sulphamethazine to be compared to the in vivo AFMU/1X ratio. The correlation was virtually perfect (r = 0.98) [Grant et al. 1990].

2.2 Cytochrome P450 2D6

Cytochrome P450 2D6 is among the most important of the CYP's because it is implicated in the metabolism of so many medicinal agents, 25% of those in common use according to Benet et al. [1996]. It constitutes the principal route of metabolic clearance for a number of drugs and therefore, variation in its expression, due to its polymorphism or other factors may entail pharmacological disturbances with serious clinical consequences.

Since the discovery of CYP2D6 polymorphism, mutations have since been identified in 15 different alleles associated with deficient or reduced activity [Sachse et al. 1997]. The polymorphism is distributed differently among races, the PM genotype being found in 5-10% of Caucasians [Duché et al. 1993], 1% of ethnic Chinese [Bertilsson et al. 1992], 1.4% of Saudi Arabians [Islam et al. 1980], 0.1% of Egyptians [Islam et al. ibid.] 0-15% of black Africans [Iyun et al. 1986; Gibson & Skett, 1994], 2-6% of black Americans [Relling et al. 1991; Marinac et al. 1995] and 5% of New Zealand Maoris [Wanwimolruk et al. 1995]. To the EM and PM classification has recently been added the very extensive metabolizer (vEM) genotype which occurs in a small percentage, 4-7%, of subjects due to gene amplification [Johansson et al. 1993] or duplication [Agúndez et al. 1995].

CYP2D6 has relatively well defined structural requirements, most substrates having an extended hydrophobic region, groups with negative potential and the ability to accept hydrogen bonds from 5-7 Å from a positively charged basic nitrogen [Smith & Jones, 1992; Strobl et al. 1993]. Substrates whose oxidation is catalysed by CYP2D6 are presented in Table 5 and include β-blockers, antiarrhythmics, antihypertensive agents and other cardiovascular
drugs. It is implicated in the biotransformation of many psychoactive compounds including antidepressants and neuroleptics.

Table 5. Substrate specificities and inhibitors of CYP2D6

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-blockers</strong></td>
<td></td>
</tr>
<tr>
<td>alprenolol, bopindolol, bufurolol,</td>
<td></td>
</tr>
<tr>
<td>metoprolol, exprenolol, pindolol,</td>
<td></td>
</tr>
<tr>
<td>propranolol, timolol</td>
<td></td>
</tr>
<tr>
<td><strong>antiarrhythmics</strong></td>
<td></td>
</tr>
<tr>
<td>encaïnide, flecaïnide, mexiletine,</td>
<td></td>
</tr>
<tr>
<td>propafenone, N-propylajmaline,</td>
<td></td>
</tr>
<tr>
<td>sparteine</td>
<td></td>
</tr>
<tr>
<td><strong>antihypertensive agents</strong></td>
<td></td>
</tr>
<tr>
<td>captopril, clonidine, debrisoquine,</td>
<td></td>
</tr>
<tr>
<td>guanoxan, indoramin</td>
<td></td>
</tr>
<tr>
<td><strong>other cardio-vascular drugs</strong></td>
<td></td>
</tr>
<tr>
<td>diltiazam, perhexilene</td>
<td></td>
</tr>
<tr>
<td><strong>antidepressants</strong></td>
<td></td>
</tr>
<tr>
<td>amiflamine, amitriptyline, clomipramine,</td>
<td></td>
</tr>
<tr>
<td>citalopram, desipramine, fluoxetine,</td>
<td></td>
</tr>
<tr>
<td>imipramine, methoxyphenamine, minaprine,</td>
<td></td>
</tr>
<tr>
<td>nefazodone, nortriptyline, paroxetine,</td>
<td></td>
</tr>
<tr>
<td>clomipramine</td>
<td></td>
</tr>
<tr>
<td><strong>neuroleptics</strong></td>
<td></td>
</tr>
<tr>
<td>clozapine, fluphenazine, haloperidol,</td>
<td></td>
</tr>
<tr>
<td>perphenazine, risperidone, thioridazine,</td>
<td></td>
</tr>
<tr>
<td>trifluperidol</td>
<td></td>
</tr>
<tr>
<td><strong>miscellaneous</strong></td>
<td></td>
</tr>
<tr>
<td>codeine, dextromethorphan, hydrocodone,</td>
<td></td>
</tr>
<tr>
<td>methoxyamphetamine, phenacetin, phendimetrazine, phenytoin,</td>
<td></td>
</tr>
<tr>
<td>ritonavir</td>
<td></td>
</tr>
</tbody>
</table>

| Non-substrates                     |                       |
|                                   |                       |
| Quinidine1                        |                       |
| Cimetidine2                       |                       |
| Dextropropoxyphene3               |                       |
| Ergotamine3                       |                       |
| Labetolol2                       |                       |
| Nicardipine2                     |                       |
| Quinine1                         |                       |
| Methadone3                       |                       |
| Chloroquine4                      |                       |

| Substrates                         |                       |
|                                   |                       |
| Fluoxetine4                       |                       |
| Paroxetine4                       |                       |
| Perphenazine4                     |                       |
| Propafenone4                     |                       |
| Thioridazine4                     |                       |
| Ritonavir5                        |                       |

| Putative non-substrates            |                       |
|                                   |                       |
| Chlorpheniramine4                 |                       |
| Cocaine4                          |                       |
| Triprolidine4                     |                       |

1From Cholerton et al. [1992]; Lamard et al. [1995]; Bertz & Granneman, [1997]; Kumar et al. [1996]. 2From Leeman et al. [1986]; Lamard et al. [1995]; Wu et al. [1993]; Bertz & Granneman [1997]; Kempf et al. [1996]; Kumar et al. [1996].
CYP2D6 is a high affinity, low capacity enzyme whose expression is not affected by classical inducers, e.g. dioxin, phenobarbital or 3-methylcholanthrene [Lamard et al. 1995]. There are no reports of induction of the isoform by any other compounds. CYP2D6 activity can be inhibited by a number of compounds especially quinidine, a single dose of which can reduce functional levels in genotypically EM subjects to those usually found only in PM's [Leeman et al. 1986]. Other inhibitors are listed in Table 5 including those shown to inhibit CYP2D6 but only in in vitro studies ("putative" in Table 5).

The most common probes of CYP2D6 activity are debrisoquine, sparteine and dextromethorphan. Bufuralol and metoprolol are no longer in common use except as in vitro probes. The antimode which distinguishes EM's from PM's is different for each probe and must be calculated from probit analysis of the metabolic quotient distribution.

In recent years dextromethorphan has become the chosen in vivo probe of most investigators studying CYP2D6. Dextromethorphan is available worldwide without prescription and is familiar to most potential subjects, many having taken it. Although adverse events associated with its ingestion are not unheard of [Schadel et al. 1995], they are exceedingly rare. Another advantage over the antiarrhythmics (debrisoquine, sparteine) is that a spot urine sample as soon as four hours after ingestion is all that is required for analysis [Ducharme et al. 1996].

2.3 Glucuronidation and Sulphation

Glucuronidation and sulphation are the most common of phase II reactions and are under the control of two families of enzymes, the UDP-glucuronosyltransferases (Table 2) and the sulphotransferases (Table 3). Effective levels of activity for both pathways are dependent upon levels of enzyme, i.e. the rate of production (transcription, translation), but also on substrate and cofactor levels. At the concentrations usually encountered
physiologically/pharmacologically, cofactor levels are more likely to be rate limiting than are levels of enzyme expression [Reinke et al. 1994].

The cofactor for glucuronidation reactions is UDP-glucuronic acid (UDPGA), synthesized through UDP-glucose from uridine triphosphate (UTP) and glucose-1-phosphate. The latter substrate may arise from glycogenolysis or from glucose-6-phosphate, yielded from the metabolism of exogenous glucose/carbohydrate. Thus glucuronidation rates reflect glycogen stores and are lowered in fasting states [Reinke et al. 1981].

The cofactor for sulphation reactions is 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Its synthesis requires two moles of ATP and inorganic sulphate or cysteine from the diet. Sulphate concentrations are rate-limiting except at very high levels [ibid.]. Unlike glucuronidation, fasting does not affect sulphation.

Miners and Mackenzie [1991] list age, certain disease states, cigarette smoking, coadministered drugs, diet, ethnicity, genetics and hormonal effects as factors which influence the activity of UDPGT. However, as Reinke et al. [ibid.] pointed out, most of these factors also affect UDPGA stores. To distinguish what mechanism produces a given change in activity is not always possible. Inducers of CYP's which have also been shown to augment glucuronidation (e.g. phenobarbital, aromatic hydrocarbons) cause increases in UDPGA levels [Goon & Klaasen, 1992]. Whether they also induce UDPGT expression is, again, difficult to determine.

Many drugs, either directly or following phase I processes, undergo both glucuronidation and sulphation (e.g. acetaminophen). Conjugation by either pathway follows first-order kinetics whereby drug concentration defines the rate of metabolism. In the case of glucuronide and sulphate formation, drug concentration also defines which conjugates are to be formed. Sulphotransferases are generally high affinity (typical $K_m$'s of 10 - 40 $\mu$M) low capacity enzymes compared with the high capacity, lower affinity ($K_m$'s of 0.1 -
0.4 mM) UDPGT's. Thus sulphates tend to be formed at low drug concentrations, glucuronides at high concentrations [Reinke et al. 1994].

Acetaminophen (APAP) is the most studied of drugs which are both glucuronidated and sulphated, in part due to the potential for hepatotoxicity from APAP overdose. Toxicity from APAP occurs only with sufficient oxidative metabolism of the compound. CYP-driven oxidation of APAP is a minor pathway which becomes important when conjugative pathways are saturated as in the case of overdose. When this occurs, metabolism of APAP by CYP's 1A2, 2E1 and 3A4 produces the electrophilic N-Acetyl-p-aminobenzoquinone imine [Thummel et al. 1993]. This metabolite in turn reacts with cellular macromolecules which ultimately causes liver necrosis.

Due to APAP's widespread use, there is continued interest in the factors which may affect APAP metabolism. Under normal conditions, where recommended doses are taken by healthy individuals, there is little if anything which alters APAP metabolism. Conjugation to glucuronic acid, sulphate and glutathione, the latter also occurring with the potentially toxic oxidative metabolite, is such that toxicity usually ensues only in cases of overdose.

However, there are unusual situations which can produce toxic effects from normal doses of APAP. For example, toxic reactions have been reported after the coadministration of isoniazid [Murphy et al. 1990] or zidovudine [Shriner & Goetz, 1992]. The former interaction was explained on the basis of isoniazid's induction of CYP2E1, the latter on the basis of competition for UDPGA. Neither explanation, on its own, has merit since APAP is normally metabolized by multiple pathways.

Alteration in the activities of the UDP glucuronosyltransferases and sulphotransferases, whether from direct inhibition (or induction) of the enzymes or from changes in cofactor availability, may be due to environmental, nutritional or disease factors. APAP has been used as a general probe of environmental effects {e.g. smoking} within healthy populations.
[Bock et al. 1987]. Heavy smoking or the coingestion of phenytoin or rifampicin were found to augment urinary APAP-glucuronide/APAP ratios but not sulphation ratios [ibid.]. Pantuck et al. [1991] showed glucuronidation of APAP, at the expense of sulphation, to be increased when calories from protein were replaced by carbohydrate in healthy men. Glucuronidation of oxazepam was also increased. Primary biliary cirrhosis has been shown to cause depressed APAP sulphation [Davies et al. 1995].

3. **HIV-infection and AIDS**

3.1 **Disease Characteristics**

HIV is a blood borne pathogen whose primary target is the helper-inducer subset of lymphocytes [Walker, 1992]. These T (thymus) lymphocytes are defined by their surface expression of the CD4+ molecule. Although only a minority of CD4+ cells are infected, HIV causes a dramatic depletion of CD4+ cells which results in the near crippling of cell mediated immune responses. The patient is left virtually defenceless against potential pathogens including microorganisms which would otherwise be harmless, leading to the so-called opportunistic infections. Patients are also at greater risk of developing tumours normally curtailed by the immune system.

HIV-associated damage to the immune system is highly variable as are the results of such damage. There are subjects with syndromes clinically identical to AIDS in whom no traces of HIV can be found. Conversely, there are subjects who have been carrying HIV for years without showing any symptoms. However, those who would claim that HIV is not the agent which causes AIDS are vanishingly few.

AIDS initially came to light as a syndrome associated with distinct population subgroups and characterized by the presence of peculiar illnesses and severe immunodeficiency. The discovery of HIV has led to most physicians thinking in terms of HIV disease covering a spectrum from
asymptomatic seropositivity through full blown AIDS [Saag, 1992]. The presence of HIV and/or severely depleted CD4+ cells in themselves do not constitute a diagnosis of AIDS. Thus AIDS is a classification, within the spectrum of HIV-related disease, more than a disease itself. In Canada patients are classified as having AIDS once they are diagnosed with an AIDS-defining illness. In the United States there is an additional criterion whereby a patient is classified as having AIDS when the CD4+ cell count falls below 200 mm$^{-3}$ irrespective of what symptoms have been exhibited. This allows access to federal aid programmes reserved for AIDS patients.

In 1993 the Centers for Disease Control (CDC) revised their system of classification to incorporate CD4+ cell count and indicator illnesses. Table 6 summarizes the classification of the 3 disease strata (columns) and CD4+ cell count strata (rows) which results in 9 possible categories of HIV disease from A1 (asymptomatic, >500 CD4+ cells/mm$^3$) through C3 (prior or current AIDS defining disease with <200 CD4+ cells/mm$^3$).

AIDS, under the revised definition in either Canada or the United States, is an assignation which is irrevocable. Patients who develop an AIDS defining illness may respond to treatment so successfully as to be in better health than patients in the CDC B category (Table 6). However, they are still classified as having AIDS and the latter patient is not.

Immunosuppression in an HIV-infected patient is most simply defined in terms of CD4+ cell count or in percent of T lymphocytes which react to antiCD4 antibodies. These CD4+ cells, the so-called T-helper cells are critical in coordinating cell-mediated immunity. Healthy seronegative subjects typically have a CD4+ titre of 800 - 1200 mm$^{-3}$ with >40% of T-cells being CD4+ cells [Walker, 1992]. HIV-infected patients are considered to be at high risk of succumbing to opportunistic infection once their CD4+ cell count falls below 200 mm$^3$ or if their CD4+ cells account for less than 20% of T-lymphocytes. That is not to say patients never develop AIDS despite fairly
high CD4+ titres or that they can't remain asymptomatic for long once their titres drop very low.

Table 6. CDC Classification Scheme of HIV Disease

<table>
<thead>
<tr>
<th>CD4+ cell category (mm-3)</th>
<th>A Asymptomatic</th>
<th>B Symptomatic (not C)</th>
<th>C AIDS defining illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. &gt;500 (≥ 29%)</td>
<td>A1</td>
<td>B1</td>
<td>C1</td>
</tr>
<tr>
<td>2. 200 - 499 (14 - 28%)</td>
<td>A2</td>
<td>B2</td>
<td>C2</td>
</tr>
<tr>
<td>3. &lt;200 (&lt;14%)</td>
<td>A3</td>
<td>B3</td>
<td>C3</td>
</tr>
</tbody>
</table>

*From Centers for Disease Control and Prevention [1992]

bNumbers in parentheses are % of lymphocytes accounted for by CD4+ cells

cConditions not included in category C either attributable to HIV infection, indicating a defect in cell mediated immunity, or whose treatment is complicated by HIV infection (e.g. oral candidiasis, persistent or resistant vulvovaginal candidiasis, cervical dysplasia, constitutional symptoms such as fever and/or diarrhoea of >1 month's duration, oral hairy leukoplakia (OHL), recurrent herpes zoster, idiopathic thrombocytopenic purpura (ITP), peripheral neuropathy.

dCandidiasis of oesophagus, trachea, bronchi or lungs, invasive cervical cancer, extrapulmonary coccidiodomycosis, cryptococcosis or histoplasmosis, cryptosporidiosis with diarrhoea of > 1 month's duration, cytomegalovirus in any organ but liver, spleen or lymph nodes, HIV-associated dementia interfering with occupation or daily living, HIV-associated wasting, Kaposi's sarcoma, lymphoma of brain, non-Hodgkin's lymphoma, disseminated mycobacterium avium or m. kansasii, pulmonary m. tuberculosis, pneumocystis carinii pneumonia (PCP), recurrent bacterial pneumonia, progressive multifocal leukoencephalopathy (PML), toxoplasmosis of internal organ, disseminated cytomegalovirus (CMV)
The AIDS epidemic has been greeted with a huge effort, on the part of the pharmaceutical industry and government, to provide new medications with which to combat HIV as well as its attendant infections and tumours. The clamour, among those infected, for speedy delivery of such treatments, has led to many drugs coming to market more quickly than might be allowed with medications for conditions perceived to be less threatening to public health. Indeed the term "fast-track" has been applied to the approval process of many such medications. It is because of such urgency that some steps which might have been expected in the approval process were curtailed. The most important of these would be the demonstration of efficacy based on clinical endpoints. Because HIV can lie dormant for years before causing AIDS, antiretroviral drugs could require too long to prove their effectiveness at preventing AIDS. Although zidovudine (AZT) was approved on the basis of survival relative to placebo, the use of surrogate markers of disease progression, especially CD4+ cell count, has become a widespread means of comparing and evaluating drug efficacy.

The value of the CD4+ cell count as an indicator of disease progression has not been universally accepted but most clinicians have been basing treatment decisions on it until recently. In fact, the expense of determining viral load (viral burden, viraemia titre) requires many to continue doing so. It is not a very sensitive index. Patients take varying lengths of time to reach a certain, arbitrary level of immunosuppression, as measured by CD4+ cell count (Table 6), after which there is further variation in timing before the onset of symptoms. This latter variation is due to a host of variables (lifestyle, nutrition, health status unrelated to HIV, genetics) including luck.

The slope of declining CD4+ cells vs. onset of serious symptoms is not very steep (i.e. sensitive) [Fauci & Rosenberg 1994]. This is in part because the range over which these disease changes take place is low: not much happens between the 800 mm$^3$ and 200 mm$^3$ levels, after which the median
time to diagnosis of an AIDS-defining illness is 12-18 months. Another factor is noise, many clinicians feeling there to be no real difference between a CD4+ cell count of 50 and 0.

Plasma viraemia titre (virions per unit of plasma) however, as measured using PCR or branched chain DNA assay, is less noisy and the slope vs. disease progression is steeper [ibid.]. It is now accepted as a viable benchmark of HIV-infection and correlates with disease progression much better than CD4+ cell count [ibid.]. One might presume that it would also better correlate with diminished capacity to metabolize drugs than did CD4+ cell count (which correlated not at all). Unfortunately CD4+ cell count was the only quantitative indicator of disease status available in the studies reported here, at least until near the very end of the recruitment period.

Some of the treatments for HIV-infection have been mentioned in passing (substrates of CYP3A4, section 1.1). It was not the aim of this research to investigate treatment effects but a brief outline of those treatments may aid the reader in understanding subsequent chapters. In terms of pharmacotherapy, there are two main objectives, to combat HIV itself and to keep in check the myriad pathogens that may infect the patient. Other problems include those unrelated to HIV, or related only indirectly (e.g. depression) but which require medication that may be affected by drugs being taken against HIV or other pathogens. The problem of HIV-related wasting will be dealt with in the next section (3.2).

Zidovudine (AZT) was the first compound registered as an antiretroviral agent and is still in wide use. It is no longer used alone to combat HIV, combination therapy having become virtually universal in developed countries. Table 7 lists the three main classes of anti-HIV drugs as well as the major examples of each. Typical combination therapy, necessary to minimize the potential for HIV resistance, would be two reverse transcriptase inhibitors and a protease inhibitor [Tsoukas, 1998]. Their administration in combination
is analogous to the practice used in cancer chemotherapy where cocktails of compounds are administered so as to avoid selecting resistant strains.

Table 7. Anti-HIV medications

<table>
<thead>
<tr>
<th>Drug type</th>
<th>Example*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoside analogue reverse transcriptase inhibitor</td>
<td>zidovudine (AZT) [48], didanosine (ddI) [11], zalcitabine (ddC) [15], stavudine (d4T) [1], lamivudine (3TC) [25], vidarabine</td>
</tr>
<tr>
<td>Nonnucleoside reverse transcriptase inhibitors</td>
<td>nevirapine, delavirdine, loviride [3]</td>
</tr>
<tr>
<td>HIV protease inhibitors</td>
<td>indinavir [4], saquinavir [10], ritonavir, nelfinavir</td>
</tr>
</tbody>
</table>

*Number of patients taking the listed medications at the time of their first metabolic assessment appear within the square brackets.

The most common initial AIDS-defining diagnosis in 1990 was PCP, defining 49% of AIDS cases. Consequently it is usual to prescribe medication against PCP prophylactically, once CD4+ cell count reaches 200 mm. The treatment of choice would be cotrimoxazole but for its high association with adverse events (Section 2.1). It is usually tried first followed by pentamidine and dapsone (with or without pyrimethamine). Atovaquone has recently been approved for PCP prophylaxis but at the same dose as for treatment.

As CD4+ cell counts fall to 100, mycobacterium avium (MAC), cytomegaloviral (CMV) and toxoplasma spp. infections increase in likelihood. Prophylactic measures include azithromycin or rifabutin, for MAC, acyclovir or gancyclovir, for CMV and dapsone-pyrimethamine for toxoplasmosis [Freedberg et al. 1998; Bucher et al. 1997].

Patients with AIDS take, on average, 7.1 different medications per month and 14% of them take more than 10 [Taburet & Singlas, 1996]. Table 8 presents the main classes of drugs prescribed in the treatment of infections.
arising during HIV infection. It does not include the drugs which many patients must also take for problems unrelated or indirectly related to HIV such as antidepressants, analgesics, cardiovascular drugs, insulin, ulcer medications etc. In a recent article, Tseng & Foisy [1997] provide a comprehensive review of potential and documented drug interactions among HIV patients which includes most of the medications in Table 8.

Tables 7 and 8 also show the number of subjects in the present study taking each medication. The average number of medications taken by the 110 subjects recruited was 3.4 and included six who were taking no medication. Antidepressants or anxiolytics and analgesics were the most commonly used drugs apart from those already mentioned. These data are mentioned here because they do not appear in any of the papers which form the body of the thesis and it reinforces the premise that to study drug metabolism in the HIV population makes good sense despite the obvious difficulties.

Table 8. Drugs used in managing infections commonly arising among patients with HIV infection

<table>
<thead>
<tr>
<th>Compound class</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>antivirals</td>
<td>acyclovir [13], foscarnet, gancyclovir, ribavirin [1], valacyclovir, vidarabine</td>
</tr>
<tr>
<td>antifungals</td>
<td>amphotericin B, fluconazole [7], itraconazole, ketoconazole [6], nystatin [3]</td>
</tr>
<tr>
<td>antimycobacterials</td>
<td>azithromycin, ciprofloxacin [4], clarithromycin, ethambutol, isoniazid, mycostatin [2], rifabutin [3], rifampicin [1]</td>
</tr>
<tr>
<td>antiprotozoals</td>
<td>atovaquone [2], cotrimoxazole [32], dapsone [5], pentamidine [8]</td>
</tr>
</tbody>
</table>

*Number of patients taking the listed medications at the time of their first metabolic assessment appear within the square brackets.*
3.2 The role of nutrition in drug metabolism and HIV-infection

Since the advent of combination therapy involving protease inhibitors, prevalence of the AIDS wasting syndrome has diminished. However, it remains a leading cause of mortality in the underdeveloped world as well as among certain populations in developed countries. Virtually all AIDS patients experience weight loss at some point in the course of their disease. Decreased lean body mass typifies wasting and still affects many with advanced AIDS. AIDS patients who have lost 20% or more of their usual body weight have median survivals of less than 2 months [Hellerstein, 1994].

Malnutrition is the leading non-HIV cause of acquired immune deficiency and can be a significant compounding condition in HIV infection. Simple starvation does not explain many features of lean body wasting in HIV infection and increasing nutrient intake or providing appetite stimulants alone is often insufficient treatment. Weight loss often reflects loss of lean tissue, but changes in other body components can mask the true state of useful lean tissue mass [ibid.].

However, malnutrition per se does not constitute nor cause wasting. Compromized nutrition is an important component of wasting and may result from reduced intake due to anorexia (e.g. due to drug effects or depression), oropharyngeal pain (candidiasis) or nausea. Malabsorption brought about by altered gut architecture due to opportunistic infections is another major cause of malnutrition in AIDS patients. However, non-AIDS patients who are starving, e.g. those with anorexia nervosa or famine victims, have a very different metabolic response to nutrient restriction.

The body's normal response to nutrient deficiency is to decrease energy expenditure and markedly reduce protein breakdown by minimizing gluconeogenesis from amino acids and excretion of nitrogenous end-products. Mobilization and oxidation of body fat are increased such that lean tissue
preservation is maximized at the expense of fat stores [ibid.]. Food is usually adequate treatment to effect complete recovery.

The key component to AIDS-related wasting, apart from malnutrition, is the lack of an appropriate response from the body. Altered metabolism, what might be called hypermetabolism, or metabolic dysregulation causes an increase in resting energy expenditure when it should decrease. Lean tissue in AIDS patients is therefore less efficiently preserved than in patients who are merely starving. Increased serum triglyceride levels are common in HIV-associated wasting, however the phenomenon is not due to increased mobilization of fat stores for energy but because of a marked increase in new fat synthesis by the liver. There is also an increase, although less marked, in gluconeogenesis and protein breakdown [Grunfeld & Feingold, 1992]. Correcting the nutrition of a wasting patient, even by parenteral means, may fail to increase lean body mass despite increased body fat. Appetite stimulants such as megestrol acetate may result in substantial but highly variable weight gain but no increase in muscle mass [Hellerstein, 1994].

The pathogenesis of wasting in AIDS is multifactorial with several processes implicated. Nutrient restriction is certainly a factor, whether due to decreased intake or decreased absorption. There is also a high correlation between wasting and infection by opportunistic organisms. However, some patients waste in the absence of any infection except HIV. Common to many infections, however, are increased cytokine levels. Cytokines act as pseudohormones to mediate immune and inflammatory responses. They include tumour necrosis factor (TNF\(\alpha\)), the interleukins and interferons. They have been shown to cause many of the irregularities of lipid metabolism seen in wasting. No single cytokine has been found to illicit wasting in animal models, however it is thought that the synergistic action of several cytokines, especially TNF\(\alpha\), interleukin-1, interleukin-6 and interferon-\(\alpha\), account for the metabolic disturbances which lead to wasting [Grunfeld & Schambelan, 1994].
The effects of cytokines on nutritional status in AIDS is controversial in that cytokine levels do not always correlate with wasting. Whatever the cause, HIV-infection and AIDS remain a leading cause of weight loss and malnutrition. It was that aspect of the syndrome that provided much of the initial impetus to pursuing this research. Malnutrition has a direct impact on drug metabolism.

Prolonged changes in diet can alter the amount and/or activity of hepatic drug-metabolizing enzymes. Deficient protein and/or energy intake can reduce enzymatic capacity within days. Changes may be insufficient to suggest malnutrition, on the basis of anthropomorphic or laboratory measurements, nor to alter standard liver function tests [Walter-Sack & Clotz, 1996].

Protein:carbohydrate ratio and total energy intake are the most important factors which affect metabolic clearance. A low carbohydrate, high protein diet reduced the half-life of both antipyrine and theophylline in healthy male volunteers; a subsequent high carbohydrate, low protein diet caused the half-lives to increase [Kappas et al. 1976]. Krishnaswamy et al. [1984] showed aminopyrine and antipyrine clearance to correlate with protein intake provided calories were adequate. As calories were restricted increasing protein intake was required to maintain the same clearance levels. In a study of otherwise healthy obese individuals, a very low calorie, protein-sufficient diet resulted in decreased oxazepam clearance but unchanged antipyrine clearance [Sonne et al. 1989]. This would seem to contradict the previous studies except that obese patients would not be short of energy. The mobilization of fat stores however, would have an impact on the availability of glucose, hence UDPGA, hence the depression of oxazepam glucuronidation but not antipyrine oxidation.

Specific nutrient deficiencies (e.g. scurvy) generally result in decreased metabolism of most drugs [Krishnaswamy, 1987]. The effect of starvation
depends on the duration and outcome: in the case of mild to moderate malnutrition in adults, the body adapts and oxidative pathways do not change or even increase as do conjugative pathways. Severe malnutrition, beyond the body's capacity to adapt and resulting in nutritional oedema (or marasmic kwashiorkor in children), leads to depressed activity of both oxidative and conjugative pathways [ibid.].

The AIDS wasting syndrome would most closely resemble the latter category of severe malnutrition. Negative nitrogen balance and inefficient use of energy typical of wasting should result in diminished capacity to synthesize the enzymes necessary for metabolism as well as restricting the supply of cofactors (e.g. UDPGA). The effect of malnutrition on drug metabolism in AIDS has not been investigated.
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CHAPTER II: Research Plan and Procedures

Appendix 1 comprises the research protocol presented to the Institutional Review Board of the Montreal General Hospital (MGH) and approved by the Ethics Committee. The purpose of the research was to ascertain the impact of HIV infection and AIDS on drug metabolism with a view toward developing the ability to assess patients on an individual basis such that treatment could be tailored to their metabolic capacity.

The methods chosen involved the administration of specific and general probe medications, chosen to reflect the \textit{in vivo} activity of specific enzymes or general conjugative processes. The analysis of urinary levels of parent and metabolite(s) led to the establishment of metabolic indices or quotients which could be compared in relation to disease progression, concomitant medication intake, nutritional status or genetic factors.

The probe drugs were dextromethorphan (DM), acetaminophen and caffeine, all available without prescription and of minuscule hazard liability. They were chosen to probe five important drug metabolizing pathways. Acetaminophen was chosen to assess glucuronidation and sulphation capacity in general (as opposed to via specific transferase isoforms). Caffeine was used to gauge \textit{N}-acetyltransferase 2 (NAT2) activity. DM served as a probe for cytochrome P450 (CYP) 2D6. The fifth pathway was to be CYP3A4, but as discussed in section 1.1 of the Introduction, DM proved inappropriate as the probe.

The patients, recruited from the Immune Deficiency Treatment Centre (IDTC) of the MGH, were approached if a previous CD4+ cell count was between 150 and 250 mm$^3$ or if CD4+ cells accounted for less than 20% of T-lymphocytes. Such a group of patients would be expected to display a spectrum of illness, characteristic of advanced immunodeficiency but including both asymptomatic patients and patients with AIDS. It was also expected that the patients would undergo disease progression over the proposed course of the study's two years. Thus a cross sectional study with a longitudinal component was conceived.

The IDTC is an outpatient clinic serving a predominately gay male population of about 1000 infected with HIV. Just over 400 patients with a CD4+ cell count between 150
and 250 mm$^3$ were identified as potential subjects from the flow cytometry lab's database. During their regular clinic visits, these patients were approached and asked to participate in the study following a brief outline of the project's goals and logistics. In all 110 patients participated. In the case of 45 patients, they were asked (and accepted) to participate more than once. Many patients agreed to participate but didn't for various reasons (e.g. they forgot) and the refusal rate was very low, less than 5%.

The logistics used to effect probe drug administration and sample collection involved the patients' being provided with a metabolic phenotyping take-home kit. The kit consisted of a brown paper bag containing the subject's copy of the informed consent (Appendix 2), a patient questionnaire (Appendix 3), a flyer containing step by step instructions and phone numbers at which to obtain additional information, two containers within Ziploc® bags in which to collect pre- and post-dose urine samples, and the probe drugs.

The probe drugs were provided as follows: The DM (30 mg) was provided as 10 mL of Robitussin DM® cough medicine (graciously donated by Whitehall-Robins Co. Inc.); the acetaminophen (500 mg) as one Atasol Forte® tablet (Frank W. Horner Inc.) and the caffeine (100 mg), supplied only to the non coffee drinkers, as one Wake-Up® tablet (Adrem Ltd.). Subjects who normally drank coffee were directed to drink a cup of coffee at the same time as taking the cough syrup and Atasol.

Subjects were asked 1) to choose a time convenient for the collection and temporary storage of samples, 2) to collect and freeze a small amount of pre-dose urine, 3) to take the three probe drugs and 4) collect and freeze a small amount of urine four hours later. The subjects were asked to keep the samples frozen until they returned to the clinic or until, according to their instructions, the samples were picked up. Most subjects chose to perform the test on the day of their return visit, collecting the pre-dose sample and taking the medications at home and collecting the post-dose sample at the clinic. Thus the storage of samples was not a problem.

Upon receipt of the urine samples, they were thawed and divided into subsamples for each drug analysis. The samples to be analyzed for caffeine metabolites were acidified
with glacial acetic acid (100 μL/3 mL) sufficient to drop the pH below 3.4. All samples were scrupulously labelled and catalogued before being stored at -20° C pending analysis. The information from the patient questionnaires was transcribed to spreadsheets and the questionnaires stored in a secure filing cabinet.

Urine samples were analyzed in batches for parent and metabolites as follows. For acetaminophen, urine was diluted and analyzed by a validated HPLC method (Appendix 5) for parent, glucuronide and sulphate conjugates (Chapter IV). Undiluted urine was analyzed for two caffeine metabolites, 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1X) by capillary electrophoresis (Chapter III). For DM and its metabolites, urine samples were extracted on solid phase cartridges, concentrated and reconstituted before analysis by HPLC (Chapter V). The two HPLC analyses allowed for the samples to be heat treated at 60° C for one hour so as to inactivate any HIV which might have been present. This was validated on the basis of superimposable chromatograms from the analysis of heated and unheated samples.

Methodology was perfected as patient samples began to accumulate. Each assay was validated so as to be assured that one probe did not interfere with the analysis of another and that storage of samples did not affect analyte levels. Control subjects were recruited in parallel so as to be able to assess the expected analyte levels and variability likely to be encountered. The timing of sample collection was addressed so as to determine the importance of compliance. Each metabolic index being based on the ratio of two analytes (e.g. glucuronide/parent for acetaminophen), timing of sample collection was found not to be very critical. Collection within one to two hours of the target proved adequate.

Each patient's medical and treatment history was compiled on a Study Report Form (Appendix 4) following perusal of the patient's medical chart. Details of medications, lab results, incidence of HIV-related and other illness, body weight and height etc. were tabulated in spread sheets and the Study Report Forms secured.

Another important aspect of the research programme was the determination of genotype for the two polymorphic pathways, NAT2 and CYP2D6. These enzymes are
under the control of genes, mutant alleles of which have been discovered and which confer reduced or absent enzyme activity. Inherited as recessive autosomal traits these diminished enzymatic capacities are found in Caucasian populations at 45-55% and 5-10%, respectively for NAT2 and CYP2D6. In healthy populations, genotype and phenotype are essentially interchangeable measurements. In ill populations however, the picture is not nearly so clear. For that reason a subset of the study's patients were selected and blood drawn. DNA was extracted and through the use of specific primers, alleles were amplified by PCR. Following digestion with specific endonucleases, the differential electrophoretic mobilities of the digests provided patterns corresponding to various alleles. Thus the genotype, to which the phenotype should respond, was determined for 61 subjects.

In summary, a total of 110 patients were entered into the study. All subjects complied but because of difficulties related to the analyses of specific samples not all subjects provided data for all pathways. Genotypes were obtained for 61 subjects. A total of 45 subjects comprised the longitudinal portion of the study. In the following three chapters the results of the studies are presented. Chapter III details the findings with respect to NAT2. Chapter IV presents the results of the acetaminophen analyses and their significance. Chapter V describes how CYP2D6 activity is affected in this HIV-infected population.
CHAPTER III: N-Acetylation among HIV positive patients and patients with AIDS: When is fast, fast and slow, slow?


The contribution of the co-authors was as follows: Dr. Brian Gilfix performed the genotype determinations on the DNA from the patients and reviewed and corrected the manuscript. Ms. Antonietta Di Girolamo provided expertise in the organization of the clinical data, helped greatly in the perusal of patients' charts for the collection of such data and recruited one quarter of the patients into the study. Dr. Christos Tsoukas provided guidance in the design of the experiments, facilitated access to the patient population and reviewed and corrected the manuscript. Dr. Irving Wainer was instrumental in the formulation of the research plan and supervised all aspects of its execution. He reviewed and revised the manuscript. William O'Neil formulated the research plan with Dr. Wainer, drafted the protocol, obtained approval for its execution from the hospital Ethics Committee, designed and prepared the consent forms, patient questionnaires, patient instruction sheets and, with the aid of Ms. Di Girolamo, the case report forms. He recruited three quarters of the patients into the study, processed all patient samples for storage, performed DNA isolation from all available patient samples and analyzed all the urine samples for caffeine metabolites by capillary electrophoresis. He drafted the manuscript and performed revisions under the direction of Drs. Wainer, Gilfix and Tsoukas.
The paper which follows describes the first demonstration of discordance between NAT2 genotype and phenotype in the same cohort of HIV positive patients and patients with AIDS. This finding had been expected based on previous genotypic and phenotypic studies done by other authors which reported a normal distribution of genotype and an abnormal distribution of phenotype, respectively, in separate HIV positive populations.

The expected distribution of phenotype (and genotype) in healthy seronegative populations of equivalent demographics would be bimodal with roughly half the subjects falling to either side of the antimode dividing slow acetylators from fast acetylators. The NAT2 phenotype distribution observed here in 105 HIV patients was unimodal and skewed toward the slow acetylator side. The distribution of genotype among a subset of 50 subjects was found to be entirely as expected, 26:24 slow:fast. Among those genotyped patients were 18 whose genotype was not reflected by phenotype: 12 slow acetylators with fast genotypes and six fast acetylators with slow genotypes.

Concomitant medication was found to bear no relation to the discrepancies between geno- and phenotype. The divergence was best explained by disease progression: among the subjects with fast genotype, the association between a slow phenotype and AIDS approached significance. Half of the fast/slow patients (6/12) had been diagnosed with an AIDS defining illness compared with only 2 of the 14 fast/fast patients. Among genotyped subjects phenotyped repeatedly were six whose phenotype "flipped." The five who went from fast to slow showed disease progression, the patient who flipped the other way showed improvement.

NAT2 genotype generally predicts phenotype in healthy populations. In this population of HIV positive patients including patients with AIDS, genotype and phenotype were found not to be interchangeable measurements of acetylating capacity especially in cases of advanced disease. Thus, to know the genotype without knowing the phenotype is of little value and vice versa.
N-Acetylation among HIV positive patients and patients with AIDS: When is fast, fast and slow, slow?

William M. O'Neil, Brian M. Gilfix, Antonietta DiGirolamo, Christos M. Tsoukas and Irving W. Wainer

ABSTRACT

Background: The discrepancy between genotype and expressed phenotype of the polymorphic N-acetyltransferase (NAT2) has been suggested by separate genotypic and phenotypic studies in populations with human immunodeficiency virus (HIV). Only one study has examined both genotype and phenotype in the same population and no discrepancies were observed.

Methods: In a cross-sectional study, 105 HIV-positive patients and patients with acquired immunodeficiency syndrome (AIDS) were phenotyped for NAT2 activity with use of caffeine as an in vivo probe; 50 of these patients were also genotyped by restriction mapping and allele-specific amplification. In a longitudinal study, 23 patients were phenotyped at least twice during the 2-year study.

Results: The distribution of the NAT2 phenotype among the 105 patients was unimodal and skewed toward slow acetylators as opposed to the bimodal distribution observed in healthy white populations. The genotype distribution was 26:24 slow:fast. There were 18 discrepancies between genotype and phenotype: 12 slow acetylators with fast genotypes and six fast acetylators with slow genotypes. No drug related effects on NAT2 activity were apparent, but the role of disease progression was evident. Among the slow acetylators whose genotype was fast the incidence of AIDS was higher (six of 12) than that among the fast acetylators whose genotype was fast (two of 14). Among patients phenotyped more than once (mean time between samples, 10.4 months) changes in phenotype from fast to slow were associated with progression of HIV infection.

Conclusions: Disease progression in HIV infection and AIDS may alter expression of the NAT2 gene. The genotype and the phenotype are not interchangeable measurements. In the HIV population, to know the genotype is useful only if the phenotype is also known and vice versa.
INTRODUCTION

The N-acetyltransferase 2 (NAT2*) locus is highly polymorphic and the various alleles have been associated with fast and slow acetylator phenotypes [Vatsis et al. 1995]. In healthy white populations the distribution of fast and slow genotypes is roughly 50:50. The \textit{in vivo} metabolic phenotype can be readily determined with use of probe drugs such as caffeine [Grant et al. 1984] and is consistent with the observed genotype distribution. In general, phenotype reflects the assigned genotype about 94\% of the time [Cascorbi et al. 1995].

However, in ill populations the relationship between genotype and phenotype is not clear. In a group of acutely ill patients with acquired immunodeficiency syndrome (AIDS), the distribution of NAT2 phenotypes was skewed toward the slow phenotype (20:8 slow:fast) compared with that observed in groups of healthy human immunodeficiency virus (HIV)-seronegative control subjects, asymptomatic HIV-positive individuals or AIDS patients without acute illness [Lee et al. 1993]. This led to the suggestion that a \textit{slow} NAT2* genotype was a risk factor in the progression of HIV infection to AIDS. A second study that examined the distribution of \textit{NAT2*} genotypes in ill patients with AIDS and in healthy control subjects found no difference in the proportion of \textit{slow:fast} genotypes [Deloménie et al. 1994]. The authors concluded that the preponderance of slow NAT2 phenotypes in acutely ill patients with AIDS was the result of an HIV-associated process that interfered with the expression of the \textit{NAT2*} gene [Deloménie et al. 1994].

To date only one study has examined both the genotype and phenotype in a single HIV-positive population [Kaufmann et al. 1996]. In the 50 patients examined there was no observable bias in the distribution of phenotype and there was near-perfect agreement between genotype and phenotype. On the basis of their findings the authors concluded that the previously reported unexpected predominance of slow NAT2 phenotypes in acutely ill patients with AIDS was attributable to drug interactions.

This article reports the results of an initial cross-sectional study, followed by a longitudinal followup, of the NAT2 activity among HIV positive patients and patients with AIDS and the relationship between \textit{NAT2*} genotypes and NAT2 phenotypes. Of 105
patients probe drug-phenotyped for NAT2 with caffeine, 50 were also genotyped by allele-specific amplification and restriction fragment length polymorphism analysis. The phenotypic distribution was unimodal and skewed toward slow whereas the genotype was distributed normally. Of the 18 discrepancies between genotypes and phenotypes, none were attributable to drug interactions and most could be associated with HIV-related disease progression.

PATIENTS AND METHODS

Study protocol and patients. The study protocol was approved by the Ethics Committee of the Montreal General Hospital. One hundred five patients with a previous CD4+ cell count between 150 and 250 mm$^{-3}$ or whose CD4+ cells accounted for less than 20% of T-lymphocytes were recruited from the Immune Deficiency Treatment Centre of the Montreal General Hospital and signed an informed consent before participating. The patients' medical records were examined thoroughly and their histories and physical examinations compiled in a standardized format on a case report form. All medications and supplements taken by the patient at the time of phenotyping were carefully recorded.

Body mass index (M/H$^2$, [mass in kilograms, height in metres]) was computed for each patient. Patients with a body mass index between 19 and 24.9 were classified as being of normal body weight. Patients below and above this range were classified as underweight and overweight, respectively [Llewellyn-Jones et al. 1984].

Phenotyping studies. All patients were assessed for N-acetylation capacity with use of caffeine as the probe. Patients were asked to abstain from caffeine during the test period. Spot urine samples were collected before and 4 hours after ingestion of 100 mg caffeine (or a cup of coffee). Urine samples were acidified with 30 μL/mL glacial acetic acid and were stored frozen at -20°C until analyzed. The ratio of 5-acetylamino-6-formylamino-3-methyluracil (AFMU) to 1-methylxanthine (1X), determined by a previously reported and validated method that uses capillary electrophoresis [Lloyd et al. 1992], was used to establish the phenotype. An area ratio of 1.0 was used as the antimode.

The first measured phenotype of each subject was included in the cross-sectional portion
of the study. To date, 23 of the 105 study participants have repeated the test at least once (mean time between samples, 10.4 mo). This constitutes the longitudinal aspect of the study.

**Genotyping studies.** Deoxyribonucleic acid (DNA) was isolated from stored lymphocytes or fresh whole blood (ethylenediaminetetraacetic acid [EDTA]) with a commercial kit (Puregene DNA Isolation kit, Gentra Systems, Minneapolis, Minn.). This extraction method allowed the introduction of a heating step (60°C for 60 min) during the cell lysis stage, providing for the inactivation of any HIV. The DNA isolated from 10⁶ lymphocytes or 300 μL blood was dissolved in 100 μL of the supplied DNA resuspension buffer.

All restriction buffers, bovine serum albumin (BSA), and restriction enzymes were from New England Biolabs, Ltd. (Mississauga, Ontario). All primers were synthesised by Sheldon Biotechnology Centre (Montreal, Quebec).

The identification of the various NAT2* alleles was performed by restriction genotyping and allele-specific amplification with a modification of the method of Doll et al. [1995]. Reactions were carried out in a volume of 50 μL that contained 5 μL (0.3 μg) of DNA; 0.2 mmol/L each of deoxyadenosine triphosphate, deoxyctydine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate; 12.5 pmol of primer NAT2-SE (5'-ATGGACATTGAAGCATAATTGGAAAGAATT-3'); 12.5 pmol of primer NAT2-AS (5'-AAGGGTTTATTTGTCCCTATTCTAAT 3'); and 5 μL of 10 x polymerase chain reaction buffer (100 mmol/L Tris hydrochloric acid, pH 9.0; 500 mmol/L potassium chloride; 15 mmol/L magnesium chloride; 2 mg/mL gelatin; and 1% Triton X100) (Vector Biosystems, Toronto, Ontario).

The DNA in the reaction mixture was first denatured for 10 min at 96°C and then cooled to 4°C. One unit of Taq polymerase (Vector Biosystems) was then added to each sample. Each sample was reheated for 2 min at 96°C and then subjected to 30 cycles in a thermal cycler, with each cycle consisting of a 10-second denaturation at 96°C, a 30-second annealing at 58°C, and a 1-minute extension at 65°C.
To identify the various alleles, 7.5 µL of the amplified material was mixed with 7.5 µL of the following:

- 1.5 µL NEBuffer 3; 0.2 µL of 10 mg/mL BSA; 0.5 µL Taqα I (20 units/µL); 0.5 µL Dra III (3 units/µL); and 4.8 µL water. The restriction mixture was incubated for 3 hours at 37°C and then for 3 hours at 65°C.
- 1.5 µL of NEBuffer 3; 0.2 µL of 10 mg/mL BSA; 0.5 µL Dde I (10 units/µL); and 5.3 µL water. The restriction mixture was incubated overnight at 37°C.
- 1.5 µL NEBuffer 2; 0.2 µL of 10 mg/mL BSA; 1 µL Msp I (20 units/µL); 1 µL Kpn I (10 units/µL); and 3.8 µL water. The restriction mixture was incubated overnight at 37°C.
- 1.5 µL NEBuffer Bam HI; 0.2 µL of 10 mg/mL BSA; 0.5 µL Fok I (4 units/µL); 0.5 µL Bam HI (20 units/µL); and 4.8 µL water. The restriction mixture was incubated overnight at 37°C.

The Fok I digestion was confirmed by the following. To 7.5 µL of the amplified material was added the 7.5 µL of a mixture that contained (1) 1.5 µL NEBuffer 4; (2) 0.2 µL of 10 mg/mL BSA; (3) 0.5 µL Fok I (4 units/µL); and (4) 5.3 µL water. The restriction mixture was incubated overnight at 37°C.

The combination of enzymes described above differs from that of Doll et al. [1995] because the Fok I/Dra III combination used in the original article was found to produce a smeared pattern on electrophoresis. In all instances, the restriction mixture was overlaid with mineral oil.

The T341C substitution was determined by allele-specific amplification. Reactions were carried out exactly as described for the initial amplification except for the use of primer NAT2-T341 (5'-CTCCTGCAGGTGACCAT-3') or primer NAT2-C341 (5'-CTCC-TGCAGGTGACCAC-3'); primer NAT2-comm (5'-GGAGACGTCTGCAGGTATG-3'). Amplification was then performed with use of a "touchdown" protocol: 20 cycles of 1 minute at 94°C, 30 seconds at 92°C, and 40 seconds at 70°C, decreasing by 0.5°C per cycle; and then 20 cycles of 40 seconds at 92°C and 40 seconds at 60°C, increasing by 1 second per cycle.
Because the allele-specific amplification method proved, in our hands, to yield high backgrounds, the T341C mutation was verified by use of the nested primer method [Doll et al. 1995]. In brief, 2.5 μL of the initial NAT2* amplicon was added to a reaction mixture as described above except for substitution of the following primers (5'-CACCTTCTCCTGCAGGTCACCG-3' and 5'-TGTCAGCAGGAATGCAA-AGGC-3'). Thermal cycling conditions were identical to that used in the initial amplification. Then, 10 μL of the amplified material were mixed with 5 μL of the following: 1.5 μL NEBuffer 3; 0.2 μL of 10 mg/mL BSA; 0.4 μL AcI I (5 units/μL); and 2.9 μL water. The restriction mixture was incubated overnight at 37°C.

**Gel Electrophoresis.** The restriction products were analyzed by electrophoresis of 10 μL of the reaction mixture on a 10% polyacrylamide gel containing TBE buffer (89 mmol/L Tris base, 89 mmol/L boric acid, and 2 mmol/L EDTA) for 2.3 hours at 70 V. The gel was then stained for 45 minutes in 0.5 μg/mL ethidium bromide, photographed under ultraviolet light, and the banding profile identified on the basis of molecular weight standards.

The products of the allele-specific amplification were analyzed by electrophoresis of 10 μL of the reaction mixture on a 1.5% agarose gel containing TPE buffer (80 mmol/L Tris phosphate and 2 mmol/L EDTA) and ethidium bromide (0.15 μg/mL) for 1 hour at 68 V. The gel was then photographed under ultraviolet light.

The analysis of the NAT2* genotype is complicated by the large number of possible alleles. Some combinations of alleles cannot be distinguished as it is not possible to know whether the mutations defining specific alleles lie on the same or opposite chromosomes without family studies (e.g. 6B/13 and 4/6A; 6A/12A and 6B/12B; 5A/12A and 4/5B). The N-acetylation activity of each allele has been classified as fast or slow on the basis of in vivo or in vitro studies [Vatsis et al. 1995]; 4, 12A, 12B, and 13 are considered to be fast; 5A, 5B, 5C, 6A, 6B, 7A, 7B, 14A, and 14B are considered to be slow; and 17 and 18 are unknown. The NAT2* genotypes are characterised as fast or slow assuming the slow alleles act recessively.
**Data analysis.** Data are presented as mean ± SD. Probit analysis consisted of plotting the log of AFMU/IX versus its probit (difference, in standard deviations, from the mean + k, where k was an integer yielding a positive probit). Curves with equations of the form \( y = ae^{bx} \) were tested for goodness of fit in an attempt to discern an antimode [Kupfer et al. 1984]. An antimode would be defined by the point at which the curves cross, so that there is complete segregation between presumed slow and fast acetylators. Odds ratios were calculated by use of Fisher's exact test and the 95% confidence interval with the approximation of Woolf (Graphpad InStat version 2.04).

**RESULTS**

**Patient characteristics.** To date, 105 patients who met the study entry criteria have participated in the study by returning at least one set of urine samples. All participants had advanced HIV-related disease, determined on the basis of moderately severe CD4+ cell depletion (mean CD4+ cell count 216 ± 141 mm\(^{-3}\)). At the time of the first phenotyping the patients were between 26 and 74 years old (43 ± 10 years). All but 14 were white subjects and the cohort included 11 women. Of the 50 patients genotyped to date, all but seven were white (there were four black American patients, one black African patient, one West Indian patient, and one Hispanic patient). The subjects, whose CD4+ mean cell count was 234 ± 167 mm\(^{-3}\) and whose age ranged from 26 - 66 years old (42 ± 9 years), included seven women. Liver function tests were within the normal range or ≤ grade 1 elevation for all but eight patients. Of these, three demonstrated grade 2 hyperbilirubinemia, three displayed grade 2 aminotransferase elevations, and one each had grade 3 elevations of bilirubin or aminotransferases.

**Cross-sectional study.** The distribution of the metabolic ratio (AFMU/IX area ratio) among the 105 study subjects is shown in Fig. 1. The population appeared as a unimodal skewed distribution, and probit analysis of the data did not yield a discernible antimode. An antimode of 1.0, previously determined in healthy volunteers, was used to assign slow or fast phenotypes [Lloyd et al. 1992]. With use of this antimode in the study population, the
proportion of slow:fast NAT2 phenotype was 2.0:1, not 1.2:1 as determined in a healthy HIV-negative population of similar demographics [Cascorbi et al. 1995].

Fig. 1. Frequency distribution of the N-acetyltransferase (NAT2) phenotype among 105 patients positive for human immunodeficiency virus (HIV). AFMU/1X, Ratio of 5-acetylamino-6-formylamino-3-methyluracil to 1-methylxanthine peak areas.

The genotypes of 50 of the patients were determined and the results are presented in Table I. The distribution of the genotypes in this group, 26 slow: 24 fast, is precisely what would be expected in a predominately white population.

In the cross-sectional population there were 18 discrepancies between genotype and phenotype, including six in which genotypically slow patients were fast acetylators. Thus four groups of patients emerged according to genotype and phenotype: fastfast (14), slowslow (18), fastslow (12) and slowfast (6).

Since it has been suggested that drug interactions may account for the observed discrepancies between phenotype and genotype [Kaufmann et al. 1996], a close examination was made of the medications each patient was taking at the time of phenotyping. There were no differences in the medications administered to the patients as...
Table 1. *N*-Acetyltransferase (NAT2) genotypes and phenotypes of 50 HIV-positive patients and patients with AIDS.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>NAT2* Alleles</th>
<th>Genotype</th>
<th>AFMU/IX</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1141</td>
<td>5B/6A</td>
<td>Slow</td>
<td>0.922</td>
<td>Slow</td>
</tr>
<tr>
<td>1142</td>
<td>4/5B</td>
<td>Fast</td>
<td>0.667</td>
<td>Slow</td>
</tr>
<tr>
<td>1145</td>
<td>5A/12A or 4/5B</td>
<td>Fast</td>
<td>1.51</td>
<td>Fast</td>
</tr>
<tr>
<td>1146</td>
<td>4/4</td>
<td>Fast</td>
<td>2.57</td>
<td>Fast</td>
</tr>
<tr>
<td>1147</td>
<td>4/5B</td>
<td>Fast</td>
<td>1.05</td>
<td>Fast</td>
</tr>
<tr>
<td>1148</td>
<td>4/4</td>
<td>Fast</td>
<td>1.87</td>
<td>Fast</td>
</tr>
<tr>
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<td>5B/5B</td>
<td>Slow</td>
<td>0.554</td>
<td>Slow</td>
</tr>
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<td>Slow</td>
</tr>
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<td>0.212</td>
<td>Slow</td>
</tr>
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<td>Fast</td>
</tr>
<tr>
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<td>4/5B</td>
<td>Fast</td>
<td>1.29</td>
<td>Fast</td>
</tr>
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<td>5B/5B</td>
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<td>Fast</td>
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<td>Slow</td>
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<td>Slow</td>
<td>0.787</td>
<td>Slow</td>
</tr>
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<td>Slow</td>
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<td>0.37</td>
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</tr>
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<td>5B/6A</td>
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<td>2.92</td>
<td>Fast</td>
</tr>
<tr>
<td>6118</td>
<td>6A/13</td>
<td>Fast</td>
<td>1.32</td>
<td>Fast</td>
</tr>
<tr>
<td>6149*</td>
<td>5A/12B</td>
<td>Fast</td>
<td>0.312</td>
<td>Slow</td>
</tr>
<tr>
<td>6151*</td>
<td>6B/13 or 4/6A</td>
<td>Fast</td>
<td>0.401</td>
<td>Slow</td>
</tr>
<tr>
<td>6152</td>
<td>5B/5B</td>
<td>Slow</td>
<td>0.29</td>
<td>Slow</td>
</tr>
<tr>
<td>6153</td>
<td>5B/5B</td>
<td>Slow</td>
<td>0.922</td>
<td>Slow</td>
</tr>
<tr>
<td>7171</td>
<td>6A/6A</td>
<td>Slow</td>
<td>0.513</td>
<td>Slow</td>
</tr>
<tr>
<td>7172</td>
<td>5B/5B</td>
<td>Slow</td>
<td>0.458</td>
<td>Slow</td>
</tr>
<tr>
<td>7184</td>
<td>6A/6A</td>
<td>Slow</td>
<td>0.182</td>
<td>Slow</td>
</tr>
<tr>
<td>7186*</td>
<td>5B/6A</td>
<td>Slow</td>
<td>1.37</td>
<td>Fast</td>
</tr>
<tr>
<td>8175*</td>
<td>6B/13 or 4/6A</td>
<td>Fast</td>
<td>0.169</td>
<td>Slow</td>
</tr>
<tr>
<td>9043*</td>
<td>5A/12A or 4/5B</td>
<td>Fast</td>
<td>0.332</td>
<td>Slow</td>
</tr>
<tr>
<td>9047*</td>
<td>4/5B</td>
<td>Fast</td>
<td>0.359</td>
<td>Slow</td>
</tr>
<tr>
<td>9048</td>
<td>5B/6A</td>
<td>Slow</td>
<td>0.687</td>
<td>Slow</td>
</tr>
<tr>
<td>9049</td>
<td>4/5B</td>
<td>Fast</td>
<td>1.3</td>
<td>Fast</td>
</tr>
<tr>
<td>9050</td>
<td>5A/12A or 4/5B</td>
<td>Fast</td>
<td>1.36</td>
<td>Fast</td>
</tr>
<tr>
<td>9052</td>
<td>5B/5B</td>
<td>Slow</td>
<td>0.787</td>
<td>Slow</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td>26</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>Slow</td>
</tr>
</tbody>
</table>

HIV, Human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; AFMU/IX, ratio of 5-acetylamin0-6-formylamino-3-methyluracil to 1-methylxanthine

*Fast genotype, Slow phenotype (n = 12) *Slow genotype, Fast phenotype (n = 6)*
grouped by geno-/phenotype. In particular the use of NAT2 and NAT1 substrates was the same: dapsone (a NAT2 substrate) was used by one patient in each of the fastslow, slowslow and fastfast groups and sulfasalazine (NAT2) was used by one of the fastslow patients. The combination product of sulfamethoxazole (a NAT1 substrate) and trimethoprim was used by 4 of 12, 3 of 18, 4 of 6 and 5 of 14 of the fastslow, slowslow, slowfast and fastfast patients, respectively.

To assess the role of disease progression in NAT2 expression, the clinical history of each patient was analyzed. The HIV infection characteristics and treatments of the 12 patients who were genotypically fast but who exhibited a slow phenotype are presented in Table II. Six of the patients had been diagnosed with AIDS at the time of phenotyping, compared with only two of the 14 patients whose fast acetylator phenotype matched their fast genotype. Among this group the odds ratio that associated a slow phenotype with AIDS compared with a fast phenotype with AIDS was 6.0 (not significant: 95% confidence interval: 0.9 - 39.2). Among the 18 patients whose slow phenotype matched their genotype, seven had had an AIDS-defining diagnosis, compared to two among the six with a fast phenotype despite a slow genotype. In this latter, genotypically slow, group the odds ratio associating a slow phenotype with AIDS compared with a fast phenotype with AIDS was 1.3 (not significant: 95% confidence interval 0.2 - 8.9).

Patients with abnormal liver function tests were distributed equally among the four genophenotype groups: three fastfast patients had grade 2 elevations, as did two fastslow patients; one of the slowfast patients displayed grade 2-3 hepatotoxicity as did two slowslow patients.

Another indicator of disease progression, body mass index, was calculated. Of subjects who were classified as underweight, 82% (9 of 11) were slow acetylators versus 66% of those who were of normal weight (38 of 58) or 64% of those who were overweight (21 of 33). After the group was divided into those who were underweight and those who were of normal weight or were overweight, the odds ratio that associated slow phenotype with underweight compared with fast phenotype was 2.4 (not significant: 95% confidence interval 0.5 - 12.0).
Table II. Characteristics of 12 genotypically fast patients displaying a slow phenotype†

<table>
<thead>
<tr>
<th>Patient code</th>
<th>AFMU/IX¢</th>
<th>NAT2* alleles§</th>
<th>Medications</th>
<th>CD4 count ( (mm^3, %) )</th>
<th>AIDS-defining diagnosis</th>
<th>Years since diagnosis (AIDS)</th>
<th>HIV-related diagnoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1142</td>
<td>0.667</td>
<td>4/5B</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>176 (11%)</td>
<td>PCP</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>1229</td>
<td>0.787</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>304 (19%)</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2110</td>
<td>0.210</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>170 (12%)</td>
<td>PCP</td>
<td>0.3</td>
<td>Oral herpes, lymphadenopathy</td>
</tr>
<tr>
<td>21130</td>
<td>0.690</td>
<td>6A/13</td>
<td>Lamivudine</td>
<td>152 (19%)</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3057</td>
<td>0.864</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>208 (13%)</td>
<td>None</td>
<td>-</td>
<td>OHL, lymphadenopathy</td>
</tr>
<tr>
<td>3206</td>
<td>0.537</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>247 (13%)</td>
<td>KSI</td>
<td>-</td>
<td>Molluscum, lymphadenopathy</td>
</tr>
<tr>
<td>3211</td>
<td>0.370</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>20 (3%)</td>
<td>None</td>
<td>-</td>
<td>Zidovudine-induced myopathy, oral candida</td>
</tr>
<tr>
<td>61497</td>
<td>0.312</td>
<td>5A/12B</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>45 (3%)</td>
<td>Wasting</td>
<td>0.1</td>
<td>Herpes zoster, oral candida, hepatitis A, microsporum</td>
</tr>
<tr>
<td>61510</td>
<td>0.401</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>84 (7%)</td>
<td>PCP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>81755</td>
<td>0.169</td>
<td>6B/13 or 4/6A</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>143 (13%)</td>
<td>None</td>
<td>-</td>
<td>Hepatitis, genital herpes, lymphadenopathy, herpes zoster, vitamin B12 deficiency</td>
</tr>
<tr>
<td>9043</td>
<td>0.332</td>
<td>5A/12A or 4/5B</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>456 (10%)</td>
<td>None</td>
<td>-</td>
<td>Oral candida</td>
</tr>
<tr>
<td>9047</td>
<td>0.359</td>
<td>4/5B</td>
<td>Lamivudine, didoxuridine, sulfamethoxazole-trimethoprim,‡‡ valacyclovir (INN, valaciclovir), versus acyclovir (INN, aciclovir), indinavir versus stavudine</td>
<td>144 (6%)</td>
<td>KS</td>
<td>4.5</td>
<td>Herpes zoster</td>
</tr>
</tbody>
</table>

PCP, Pneumonia caused by pneumocystis carinii; KS, Kaposi's sarcoma; OHL, oral hairy leukoplakia.
††Patients aged 29 - 54 years (mean age, 41.8 ± 7.9 years).
¶Antimode = 1.0.
§§fast alleles are 4, 12A, 12B and 13; slow alleles are 5A, 5B, 6A and 6B; fast alleles are dominant.
○Indicates female patients.
¶¶Mixed race patient; ‡‡Black American patient (all others were white patients).
‡‡NAT1 substrates
§§NAT2 substrates
©©Active at the time of phenotyping.
††Additional AIDS-defining diagnoses of gastric lymphoma and non-Hodgkin's lymphoma of parotid.

Longitudinal study. The probe drug-determined NAT2 phenotypes of 23 patients were followed longitudinally. Changes of phenotype were observed in 11 patients: five from fast to slow, five from slow to fast and, in one patient, phenotyped three times, fast to slow to fast. Table III presents the clinical findings for the longitudinal participants for whom the
genotype has been determined. Table IV presents the clinical findings for the longitudinal participants for whom the genotype has yet to be determined.

Table III. Relationship between clinical status and NAT2 phenotype among 12 patients of known genotype—longitudinal data

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Genotype</th>
<th>Changes in clinical findings between phenotypings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1148</td>
<td>Fast</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KS remained stable, oral candida recurred, onychomycosis subsided, molluscum and condyloma developed</td>
</tr>
<tr>
<td>3206</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unresponsive active KS responded to treatment and stabilized, molluscum infection subsided, lymphadenopathy greatly lessened</td>
</tr>
<tr>
<td>1230</td>
<td>Fast</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HSV remained active, constitutional symptoms increased</td>
</tr>
<tr>
<td>3211</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zidovudine-induced myopathy and oral candida resolved</td>
</tr>
<tr>
<td>6151</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>History of PCP, developed cytomegalovirus cholangitis (active at second phenotyping), CE, thrombocytopenia and liver failure; developed cytomegalovirus retinitis and neutropenia (third phenotyping)</td>
</tr>
<tr>
<td>2141</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral candida (active at first phenotyping), stable KS and recent mycobacterium gordonae infection; disseminated KS with second-degree lymphoedema (second phenotyping), wasting, bacterial pneumonia, increased constitutional symptoms and chronic fungal skin rash</td>
</tr>
<tr>
<td>3052</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remained asymptomatic but became hypersensitive to sulfamethoxazole-trimethoprim (second phenotyping)</td>
</tr>
<tr>
<td>6115</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>One HIV-related diagnosis (OHL, first phenotyping), two AIDS-defining diagnoses (wasting, PCP, second phenotyping), with hepatitis and zidovudine-induced fatty liver that continued through the third phenotyping.</td>
</tr>
<tr>
<td>2143</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remained asymptomatic through the first two phenotypings, oral candida developed by the third.</td>
</tr>
<tr>
<td>3055</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KS remained stable, no recurrence of HSV, neutropenia and oral candida developed (resolved by second phenotyping)</td>
</tr>
<tr>
<td>3205</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remained stable (controlled KS)</td>
</tr>
<tr>
<td>7184</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Active PCP, history of wasting syndrome and had recurrent infections (unresponsive rhodococcus, HZ, candida) and adverse drug reactions, pancreatitis eventually developed.</td>
</tr>
</tbody>
</table>

KS, Kaposi’s sarcoma; HSV, herpes simplex viral infection; PCP, pneumocystis carinii pneumonia; CE, candidiasis esophagitis; OHL, oral hairy leukoplakia; HZ, herpes zoster infection.

*Phenotypes measured between 6 and 14 months apart (10.7 ± 2.2)
A relationship between disease progression and discordance from genotype (e.g. genotypically fast patients with slow phenotype) or a change in phenotype (patients whose phenotype "flipped" from fast to slow) was evident among the patients for whom the genotype was known (Table III). Of the five patients who flipped from fast to slow (patients 1148, 2141, 3052, 6115, and 2143), all experienced at least some progression of disease. However, patient 3206, whose phenotype reverted to that expected from the genotype, showed improvement: his Kaposi’s sarcoma ceased to progress and tumour reduction occurred in the lower limbs to the point where he no longer required a cane to walk. Of patients whose phenotype did not change, disease progressed in some (the fastslow 6151, the slowslow 7184) but not in others.

Table IV. Relationship between clinical status and NAT2 phenotype among 11 patients of unknown genotype—longitudinal data

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Phenotype^</th>
<th>Changes in clinical findings between phenotypings</th>
</tr>
</thead>
<tbody>
<tr>
<td>9045</td>
<td>Fast Slow</td>
<td>Active CE, recurrent diarrhea and HSV, history of anal condyloma (first phenotyping), penile condyloma and neutropenia developed (resolved by second phenotyping)</td>
</tr>
<tr>
<td>2113</td>
<td>Slow Fast</td>
<td>Asymptomatic (first phenotyping), active CE, PCP and orogenital HSV developed (second phenotyping)</td>
</tr>
<tr>
<td>2118</td>
<td>Slow Fast</td>
<td>Remained asymptomatic</td>
</tr>
<tr>
<td>3207</td>
<td>Slow Fast</td>
<td>Remained asymptomatic</td>
</tr>
<tr>
<td>7173</td>
<td>Slow Fast</td>
<td>No recurrence of oral candida</td>
</tr>
<tr>
<td>1228</td>
<td>Fast Fast</td>
<td>No increase in constitutional symptoms</td>
</tr>
<tr>
<td>2115</td>
<td>Fast Fast</td>
<td>Active herpes zoster and hepatitis B infections (first phenotyping) resolved, neutropenia developed (second phenotyping)</td>
</tr>
<tr>
<td>9050</td>
<td>Fast Fast</td>
<td>History of neutropenia and peripheral neuropathy resolved by first phenotyping, PCP and CE developed by second phenotyping</td>
</tr>
<tr>
<td>2119</td>
<td>Slow Slow</td>
<td>History of folliculitis, bacterial pneumonia, onychomycosis, had rash from sulfamethoxazole-trimethoprim at first phenotyping, no further findings at second phenotyping</td>
</tr>
<tr>
<td>3051</td>
<td>Slow Slow</td>
<td>History of wasting and CE, no changes</td>
</tr>
<tr>
<td>9054</td>
<td>Slow Slow</td>
<td>History of OHL, oral candida (active at first phenotyping) PCP developed (active at second phenotyping)</td>
</tr>
</tbody>
</table>

CE, candidiasis esophagitis; HSV, herpes simplex viral infection; PCP, *pneumocystis carinii* pneumonia; OHL, oral hairy leukoplakia.

^Phenotypes measured between 3 and 14 months apart (10.5 ± 3.7).
The relationship between disease progression and change in phenotype among the 11 patients for whom genotype has yet to be determined (Table IV) is less clear. Most (8 of 11) showed little if any change, whereas the other three patients progressed to AIDS while their phenotype stayed the same or went from slow to fast.

DISCUSSION

This study was designed to detect differences in drug metabolism among HIV-positive patients and patients with AIDS in a longitudinal fashion. The initial selection criterion was a CD4+ cell count of 150 - 250 mm$^3$ (or <20% of lymphocytes) at which point advanced HIV-related disease occurs; however, some patients will still be in an asymptomatic state. Many of the individuals will have AIDS-defining events and are thus an ideally suited population to study in terms of the use of multiple medication, HIV infection activity, and the onset of new opportunistic infections or tumours. Some patients, selected to participate on this basis, had undergone significant changes in CD4+ count by the time they were recruited and participated; hence the inclusion of some subjects occurred outside the target range.

The distribution of the phenotype among the 105 patients (based on the first phenotype determination) and the lack of bimodality are consistent with the conversion of many patients from a fast to a slow phenotype: the failure of roughly half of the fast genotypes to express the fast phenotype. This is borne out on examination of the data from the group of patients who have been genotyped so far: 12 of the 24 genotypically fast patients (precisely half) were phenotypically slow.

Many factors may play a role in changing one's capacity to acetylate. Concomitant medication seems to be an obvious candidate in such a population because so many drugs are involved, although there are no known inhibitors nor inducers of NAT2. Indeed, Kaufmann et al. [1996] concluded that drug interactions are the main reason for the results observed by others [Lee et al. 1993; Deloménie et al. 1994]. However, our data suggest no such role. The use of known NAT1 and NAT2 substrates (as well as all other drugs) was equivalent across the four genophenotype groups.
Disease progression can be considered to be a possible cause of diminished acetylation capacity. Among the 105 subjects viewed cross-sectionally, surrogate markers such as CD4+ count bore no relation to phenotype as has been observed previously [Kaufmann et al. 1996]. Neither did there appear to be much correlation between incidence of slow phenotype and Centers for Disease Control disease stratum [Centers for Disease Control and Prevention, 1993] (63%, 57% and 68% of class A, B and C subjects, respectively, were slow acetylators). However, if classes A and B are grouped as being "nonAIDS," the odds ratio that associates slow phenotype with AIDS compared with fast phenotype was 0.42 (95% confidence interval 0.18 - 0.96 - significant). No other disease processes (e.g. hepatotoxicity) present themselves as culprits in the disturbance of acetylation pathways.

Low body mass index or weight loss, an indicator of disease progression [Kotler, 1994], provided some correlation among the 105 subjects viewed as a whole, inasmuch as 82% of the underweight subjects were slow acetylators versus 66% and 64% of those who were of normal weight or were overweight, respectively, although this trend did not reach statistical significance. That malnutrition per se causes a perturbation in acetylation may seem doubtful on the basis of published reports that showed an undernourished cohort of subjects displayed the same phenotype distribution as did a well-nourished one [Shastri, 1982]. However, unlike simple malnutrition, AIDS-associated wasting is characterized by a hypermetabolic state that includes serious disruption of carbohydrate and lipid metabolism [Hellerstein et al. 1990]. This disruption could lead to diminished pools of cofactors, such as acetyl coenzyme A, with a concomitant loss of NAT2 activity. A larger study population, using a more sophisticated method for measuring body cell mass (lean body mass), would be required to verify this hypothesis.

If one focuses on the 50 genotyped patients, the role of disease state in genotype expression is apparent. Among those subjects with fast genotypes, one finds six patients with AIDS among the 12 slow acetylators versus two patients with AIDS among the 14 phenotypically fast acetylators. Among the genotyped patients there were six whose phenotype was fast despite a slow genotype. Although this discrepancy was not expected, it has been reported previously [Cascorbi et al. 1995]. Among 563 white individuals
genotyped and phenotyped for NAT2, discrepancies of the slowfast type (23 of 563) were seen as well as fastslow (15 of 563) [Cascorbi et al. 1995]. One of the fastslow subjects (2143) had a metabolite ratio of 1.18. This subject, along with 13 others, lies within the "grey area" between slow and fast. In a healthy population, a small percentage of subjects are likewise found to be too near the antimode to be reliably phenotyped. In our unimodal skewed population, that fraction is higher.

NAT2 is generally considered to be non-inducible in man, although glucocorticoids have been shown to enhance in vivo acetylation rates in both rabbits [du Souich and Courteau, 1981] and rats [Zaher and Svensson, 1994]. Thus the possibility of a metabolic basis for overexpression of NAT2 cannot be precluded. Indeed, augmented blood glucose levels have been reported to magnify acetylation capacity [Przemyslaw et al. 1994]. In HIV infection, lipid metabolism is disrupted leading to hypertriglyceridemia and increased fatty acid levels in the liver [Grunfeld et al. 1992]. This could in turn lead to enhanced acetylation capacity on the basis of larger cofactor pools. However, the process may eventually result in fatty infiltration of the liver and decreased acetylation. The existence of an unknown, compensating mutation might also explain the phenomenon of slowfasts.

Of the six discrepant slow genotypes, four were phenotyped on subsequent occasions and all of them reverted to a slow phenotype (one, however, was fast again when phenotyped a third time). Disease progression was associated with two of the reversions (2141 and 6115; Table III), whereas hypersensitivity to sulfamethoxazole-trimethoprim developed in a third patient. This latter finding is, in a way, more consistent with published reports [Carr et al. 1994] than with our own data. In our study, patients who had failed sulfamethoxazole-trimethoprim treatment due to hypersensitivity reactions were no more likely to express a slow phenotype (80%) than were patients who had never tried the drug (77%). Among patients taking the medication at the time of phenotyping, 48% were slow. However, because the phenotype can change, the timing of its determination is important if it is to be used in assessing risk to hypersensitivity.

That a fast acetylator should become slow after becoming ill or malnourished or after taking numerous medications is understandable and, based upon the literature, expected
[Cascorbi et al. 1995, Lee et al. 1993]. For the most part the longitudinal data collected so far bears this out. Table III shows the disease status of the genotyped patients who were phenotyped more than once. Those with a fast genotype showed marked disease progression associated with a uniformly slow phenotype (6151) or slight progression in two subjects, one who went from fast to slow (1148) and one who stayed fast (1230). An improvement of the status of patient 3206 was associated with a reversion of phenotype to reflect genotype.

Table IV shows the disease status of the remainder of the patients who were phenotyped more than once. These included one patient whose phenotype went from fast to slow and whose disease progressed slightly and four who went from slow to fast and included one who progressed from the asymptomatic stage to AIDS. This group of ungenotyped patients included six whose phenotype did not change. Of the two phenotypically unchanged patients that showed marked disease progression there was one who stayed slow and one who stayed fast. The remainder, who showed little or no change, were also split evenly between those who stayed slow and those who stayed fast. It is difficult to explain these findings without knowing the genotype.

In conclusion, the NAT2 phenotype is expected to follow the genotype, but this relationship can be altered. Our results fail to implicate metabolic drug interactions in any of the observed perturbations of N-acetylation. Disease progression in HIV infection and AIDS emerges as the strongest candidate for altering gene expression. Wasting may also contribute to this effect but further work is required to establish the relationship.

What also emerges from this study is that the genotype and the phenotype are not interchangeable measurements. In a healthy population, one generally predicts the other. However, in the HIV population, knowledge of the genotype is useful only if the phenotype is also known and vice versa. In this population, the relationship between genotype and phenotype, and the changes in this relationship, may prove to be an important clinical marker of disease status.
Although induction of NAT2 activity by small organic molecules has not been reported, the immunostimulants interferon-γ and streptolysin O have been shown to up-regulate NAT2 activity in rats by 120% and 130%, respectively [Walter et al. 1996]. Although the mechanism(s) of the observed induction has not been elucidated, interferon-γ- and streptolysin O-mediated pretranslational or posttranslational effects have been suggested. Because elevated plasma levels of endogenous interferon [Mildvan et al. 1992] have been associated with HIV+/AIDS infection and pathogenesis, it is possible that this may be the source of the discordance between patients with a slow NAT2 genotype and fast NAT2 phenotypes observed in this study. The relationship between interferon and other cytokine levels and NAT2 genotype/phenotype is currently under investigation.

References


CHAPTER IV: Glucuronidation and sulphation of paracetamol in HIV positive patients and patients with AIDS

This paper, by W.M. O’Neil, J.C. Pezzullo, A. Di Girolamo, C.M. Tsoukas and I.W. Wainer, was submitted to Br. J. Clin. Pharmacol. in February, 1998. At the time of final submission it has been revised for resubmission to the journal.

The contribution of the co-authors was as follows: Dr. Pezzullo performed the statistical analysis and reviewed and corrected the manuscript. Ms. Antonietta Di Girolamo provided expertise in the organization of the clinical data, helped greatly in the perusal of patients' charts for the collection of such data and recruited one quarter of the patients into the study. In addition, she refined and validated the HPLC assay and performed about half of the urine analyses for acetaminophen and metabolites. Dr. Christos Tsoukas provided guidance in the design of the experiments, facilitated access to the patient population and reviewed and corrected the manuscript. Dr. Irving Wainer was instrumental in the formulation of the research plan and supervised all aspects of its execution. He reviewed and revised the manuscript. William O’Neil formulated the research plan with Dr. Wainer, drafted the protocol, obtained approval for its execution from the hospital Ethics Committee, designed and prepared the consent forms, patient questionnaires, patient instruction sheets and, with the aid of Ms. Di Girolamo, the case report forms. He recruited three quarters of the patients into the study, processed all patient samples for storage, developed (with Ms. Di Girolamo) the HPLC assay and analyzed about half of the urine samples for acetaminophen and metabolites. He drafted the manuscript and performed revisions under the direction of Drs. Wainer and Tsoukas.
The following paper describes the interindividual variation in the phase II processes of glucuronidation and sulphation found among the HIV positive patients. Unlike the distribution of NAT2 activity, nothing unusual, beyond wider variation, was seen in the distribution of either pathway's activity.

A recent report in the literature had reported HIV-related disease to have an impact on the two pathways, causing decreased glucuronidation and increased sulphation. No such effect was revealed here. Disease state, active AIDS vs. asymptomatic HIV positive vs. control, bore no relation to either glucuronidation or sulphation. The longitudinal component of the study also showed disease progression to have no significant effect on the calculated acetaminophen- glucuronidation and sulphation indices.

The concomitant administration of dapsone appeared to significantly affect sulphation in the cross sectional section of the study. In the longitudinal portion of the study, zidovudine appeared to decrease glucuronidation. Glucuronidation appeared to be lower among underweight patients.

The conclusions to be drawn from this paper are that glucuronidation and sulphation are robust pathways which do not easily succumb to the environmental forces brought to bear during HIV infection. The paper also demonstrates the validity of a simplified procedure for the in vivo assessment of the pathways which involves a 4-hour spot urine sample.
Glucuronidation and sulphation of paracetamol in HIV positive patients and patients with AIDS
William M. O'Neil, John C. Pezzullo, Antonietta Di Girolamo, Christos M. Tsoukas and Irving W. Wainer

ABSTRACT
The effect of disease state and disease progression in 108 HIV-positive patients and patients with AIDS on the glucuronidation and sulphation of paracetamol (APAP) was investigated. The extent of APAP-glucuronidation and APAP-sulphation was assessed using a spot urine sample collected 4h after the oral administration of 500 mg of APAP. The molar concentrations of APAP and its glucuronide and sulphate metabolites were determined using a validated HPLC method and APAP-glucuronidation and APAP-sulphation metabolic indices were constructed using APAP-metabolite/APAP molar concentration ratios. No effect of disease state, AIDS vs. asymptomatic HIV positive vs. control, on APAP glucuronidation or sulphation was observed. The patient population was studied over time and disease progression also did not significantly alter the calculated APAP-glucuronidation and APAP-sulphation indices. The effect of the concomitant administration of other therapeutic agents was assessed and in the cross sectional portion of the study dapsone appeared to significantly decrease APAP-sulphation as did lamivudine. In the longitudinal portion of the study the latter effect was not observed but zidovudine was seen to increase APAP-glucuronidation. The data also indicates that APAP-glucuronidation may be reduced in patients who are >10% below their ideal body weight.

1. Introduction
Clinically observed differences in drug disposition and effect are often the result of inter-individual variations in drug metabolism. These variations can produce clinically important effects [Eichelbaum et al. 1992]. For example, HIV-positive patients identified as having a slow N-acetyltransferase 2 (NAT2) metabolic phenotype are prone to hypersensitivity to the drug sulfamethoxazole [Deloménie et
al. 1994] while individuals possessing a poor cytochrome P450 (CYP) isoform CYP 2D6 phenotype react poorly to a number of psychotropic drugs [Köhler et al. 1997].

The genetic bases for the differentiation of humans into fast or slow NAT2 metabolic phenotypes [Doll et al. 1995] and efficient or poor CYP2D6 metabolic phenotypes [Evans et al. 1993] have been identified, as have the sources of a number of other polymorphic enzymatic pathways [Stubbins et al. 1996]. Assays utilizing allele-specific amplification and restriction fragment length polymorphism analysis are now routinely used to assign metabolic genotypes [Doll et al. ibid.]. In addition, probe drugs can be used for in vivo phenotypic assessments [Evans et al. 1989; Kalow et al. 1996]. For example, dextromethorphan has been used to probe CYP2D6 activity [Evans et al. 1989] and caffeine is an ideal probe for NAT2 [Tang et al. 1991]. In healthy individuals, there is a concordance between genetic and probe drug determined metabolic phenotypes for CYP2D6 and NAT2 [Funck-Brentano et al. 1992; Cascorbi et al. 1995].

However, the level of expressed metabolic activity can be affected by environmental factors such as the concomitant intake of multiple medications and/or other xenobiotics [Rajaonarison et al. 1992] as well as nutritional status and diet [Rikans, 1986; Hathcock, 1985]. A recent study of NAT2 activity in a group of 105 HIV-positive patients and AIDS patients has also demonstrated that disease state can affect enzymatic activity [O'Neil et al. 1997]. The fact that a discordance between NAT2 genotype and expressed NAT2 phenotype was observed in a cohort of HIV-positive and AIDS patients should not be surprising since this population requires multiple medications to treat myriad illnesses, many of which have an impact on nutrition.

Paracetamol (APAP) is another agent whose metabolism and disposition may be altered in HIV-positive and AIDS patients. The primary metabolic disposition of APAP occurs through glucuronidation and sulphation by UDP glucuronosyltransferase and sulphotransferase, respectively [Cummings et al. 1967]. Zidovudine (AZT) is also cleared primarily by glucuronidation, and Shriner and Goetz [1992] have
reported severe APAP-induced hepatotoxicity in an AIDS patient who was also receiving AZT. A competition for glucuronic acid cofactor was suggested as a potential cause of reduced APAP clearance and, thereby, increased hepatotoxicity. However, it has been shown in vitro [Kamali & Rawlins, 1992; Ameer et al. 1992] that different isoforms of UDP glucuronosyltransferase are responsible for the glucuronidation of AZT and APAP, and Burger et al. [1994] found no changes in the pharmacokinetics of either drug when coadministered. Metabolic drug interactions would therefore be unexpected.

Another possibility is an alteration in the activities of the UDP glucuronosyltransferases due to environmental or disease factors. Indeed, APAP has been used as a general probe of environmental effects (smoking) within a healthy population [Bock et al. 1987]. An effect of disease status on APAP metabolism has also been suggested by Esteban et al. [1997], who report that the excretion of APAP-glucuronide, expressed as percent of APAP dose, was decreased, and that of the APAP-sulphate conjugate increased in AIDS patients relative to HIV-positive patients without AIDS or controls.

This article presents the results of a larger study of paracetamol metabolism in the HIV-infected population that includes a longitudinal component.

2. Methods

2.1 Study protocol and subjects:

The study protocol was approved by the Ethics Committee of the Montreal General Hospital (MGH). Patients were recruited from the hospital's Immune Deficiency Treatment Centre on the basis of a previous CD4+ cell count between 150 and 250 mm$^{-3}$, or if CD4+ cells accounted for less than 20% of T-lymphocytes. The 108 study participants signed an informed consent before participating. The patients' medical records were examined thoroughly and their history and physical exam compiled on a case report form; all medications and supplements taken by the patient at the time of testing were
carefully recorded. Control subjects were recruited from among laboratory personnel, family and acquaintances, and signed informed consent before participating. A questionnaire was used to collect details of medication, alcohol, tobacco and caffeine intake in controls and patients. Ideal body weight was calculated from height according to Devine [1974].

2.2 Metabolism studies:

The glucuronidation and sulphation capacities of the subjects were assessed using APAP as the probe. Spot urine samples were collected 4 hours after ingestion of one 500 mg APAP tablet (Atasol Forte®, Frank W. Horner Inc., Montreal, QC, Canada) and the urine samples were stored at -20°C until analyzed. No analyte showed appreciable degradation at -20°C over 6 months [Di Girolamo et al. 1998]. The molar urinary APAP, APAP-glucuronide and APAP-sulphate concentrations were determined by a validated HPLC assay [ibid.] which provided a limit of quantitation for APAP of 5 μg/mL. LOQ for the metabolites was not determined due to their high urinary concentration.

In order to validate the use of a 4-hour spot urine sample, six subjects performed the test at least 3 times. In addition, during one of the determinations, three of the repeat controls collected extra samples at 2, 3, 5 and 6 hours after ingesting the tablet. These three subjects were also tested in triplicate within one week, during which 0-8 hour urine samples were collected.

2.3 Data analysis:

Data are presented as mean ± SD. The distributions of glucuronidation and sulphation ratios were found to be significantly non-normally distributed (p < 0.001 by Kolmogorov-Smirnov test), so non-parametric analyses were used whenever possible. A square-root transformation was found to remove most of the skewness and to bring the distributions sufficiently close to normality (by Q-Q plot) to justify the use of multivariate regression and analysis of variance (ANOVA) when appropriate. One way ANOVA among subgroups was followed, where significant differences were found, by the Tukey-Kramer
multiple comparison test provided that Bartlett's test for homogeneity of variances was not significant. Failure of Bartlett's test led to the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test (Graphpad InStat ver. 2.04). Two-group comparisons employed Student's t-test or the Mann-Whitney (non-parametric) test. One-way ANOVA was used to assess the dependence of APAP conjugation on AIDS classification; this was followed by specific contrasts to compare controls with HIV-positives, asymptomatic with symptomatic, and non-AIDS with AIDS patients. Odds ratios were calculated using Fisher's exact test and the 95% confidence intervals were obtained by the approximation of Woolf (Graphpad, InStat, ver. 2.04).

Multivariate regression analysis was applied to determine if concomitant medications had an impact on APAP conjugation. Each of the 15 most commonly taken medications was coded as 1 if the patient were taking that medication and 0 otherwise. A similar process was applied to alcohol, caffeine and tobacco use in which the level of consumption was graded: e.g. Patients who had never smoked were graded -1, former smokers, light smokers (< 10 cigarettes per day) and heavy smokers were graded 0, 1 and 2 respectively.

For longitudinal analysis, changes in the medication during the interval were coded to distinguish among four situations with respect to each medication: never being on the medication during the study, going onto the medication during the interval, discontinuing medication during the interval, and remaining on the medication for the entire time of the study. This four-way classification was used to perform two kinds of tests: one to determine, for each medication, whether patients in any of the four groups experienced significant changes in glucuronidation or sulphation ratios during the interval, and another to determine whether the amount of change in the ratios varied significantly among the four groups.
3. Results

3.1 Subject characteristics:

Of the 108 patients studied, 11 were female and all but 14 were Caucasian. Ages ranged from 26 and 74 years (43 ± 10 years). All patients recruited into the study met the initial CD4+ criteria of a cell count between 150 and 250 CD4+ mm$^{-3}$. During the 4-8 week period between recruitment and initial phenotyping there were a number of significant fluctuations in individual CD4+ cell counts, so at the initiation of the study the CD4+ cell counts ranged from 0 to 1029 mm$^{-3}$ (215 ± 140; mean ± SD). Liver function tests were normal, with only eight patients exceeding a grade 1 elevation. Of these, three demonstrated grade 2 hyperbilirubinaemia, three displayed grade 2 aminotransferase elevations, and one each had grade 3 elevations of bilirubin or aminotransferases. Forty-six subjects were tested more than once, the mean time between the 1$^{st}$ and 2$^{nd}$ determinations being 12.6 months.

At the time of the first determination 24 patients were asymptomatic (Centers for Disease Control [1992] disease stratum A) and 41 had AIDS (CDC stratum C). The remaining 43 patients, having been diagnosed with an HIV-related but not AIDS-defining disease, were classified as CDC stratum B.

Of the 36 study participants recruited to serve as controls, 14 were female, and all but 6 were Caucasian; ages ranged from 19 to 68 (37 ± 11 years). Four subjects were measured at least 3 times, up to 60 days apart.

3.2 Probe validation:

Previous studies of the intersubject variability of APAP conjugation [Bock et al. 1987] were based on the analysis of a 0-8 hour urine sample. In order to validate the use of a 4-hour spot sample, three control subjects participated in the test at least 6 times. On four or more occasions the subjects provided a urine sample four hours after administration of APAP, and on three other occasions the total 0-8h urinary excretion was collected. Glucuronidation and sulphation ratios in the 4h and 0-8h samples were calculated by dividing the molar
concentration of APAP-glucuronide and APAP-sulphate by the molar concentration of APAP. There were no significant differences in the calculated glucuronidation and sulphation ratios determined in the 4h and 0-8h samples, Table 1.

Table 1: Variability in Paracetamol Glucuronidation and Sulphation Ratios with Time of Sample Collection among Control Subjects

<table>
<thead>
<tr>
<th>Time (h) Post-dose</th>
<th>Glucuronidation Ratio</th>
<th>Sulphation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Subject</td>
<td>Subject</td>
</tr>
<tr>
<td>Mean 4-h</td>
<td>23.0 27.8 35.4</td>
<td>8.74 6.93 14.5</td>
</tr>
<tr>
<td>SD</td>
<td>9.91 8.12 10.2</td>
<td>2.54 2.24 4.51</td>
</tr>
<tr>
<td>Mean 0-8 h</td>
<td>21.9 26.2 30.6</td>
<td>8.61 7.72 15.1</td>
</tr>
<tr>
<td>SD</td>
<td>7.26 4.76 7.95</td>
<td>1.59 1.87 3.82</td>
</tr>
</tbody>
</table>

In order to assess the impact of urine collection time on the metabolic ratios, i.e. compliance to the study protocol, during the same experiments where 4h samples were collected, the subjects also provided samples at 2, 3, 5 and 6 hours post-dose. No significant differences were observed in the calculated glucuronidation and sulphation ratios determined in the 3h, 4h, 5h and 6h samples, Figure 1. The results indicate that a variation of ± 1h in the time of sampling should not affect the determination of the metabolic ratios.

The molar concentration ratio of metabolite (either glucuronide or sulphate):parent was measured in the 4-hour urine samples to establish metabolic activity indices of capacity. In order to assess inter-day variations in the glucuronidation and sulphation ratios, 5 control subjects were evaluated on 3-8 nonconsecutive days. The relative range (range/mean) and maximum and minimum inter-day values of the glucuronidation and sulphation ratios are presented in Table 2. The results indicate that up to a 3-fold inter-day variation in an individual's glucuronidation or sulphation ratio should not be considered as meaningful.
Figure 1: Variation in Spot Urine Paracetamol Molar Glucuronidation (A) and Sulphation (B) Ratios over Time
Table 2: Variability within Control Subjects in Their Capacity to Glucuronidate and Sulphate Paracetamol Compared to That within the Control Population

<table>
<thead>
<tr>
<th>Subject I.D. Determinations (n)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>Glucuronidation Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>23.0</td>
<td>27.8</td>
<td>19.2</td>
<td>25.0</td>
<td>35.4</td>
</tr>
<tr>
<td>SD</td>
<td>9.91</td>
<td>8.12</td>
<td>1.21</td>
<td>4.34</td>
<td>10.2</td>
</tr>
<tr>
<td>MAX</td>
<td>36.2</td>
<td>35.9</td>
<td>20.4</td>
<td>32.6</td>
<td>49.0</td>
</tr>
<tr>
<td>MIN</td>
<td>13.0</td>
<td>14.0</td>
<td>18.0</td>
<td>19.2</td>
<td>27.1</td>
</tr>
<tr>
<td>RSD %</td>
<td>43.1</td>
<td>29.2</td>
<td>6.30</td>
<td>17.4</td>
<td>28.8</td>
</tr>
<tr>
<td>Relative Range %</td>
<td>101</td>
<td>78.8</td>
<td>12.5</td>
<td>53.6</td>
<td>61.9</td>
</tr>
<tr>
<td>MAX/MIN</td>
<td>2.78</td>
<td>2.56</td>
<td>1.13</td>
<td>1.70</td>
<td>1.81</td>
</tr>
<tr>
<td>Sulphation Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN</td>
<td>8.74</td>
<td>6.93</td>
<td>10.5</td>
<td>14.1</td>
<td>14.5</td>
</tr>
<tr>
<td>SD</td>
<td>2.54</td>
<td>2.24</td>
<td>1.73</td>
<td>4.04</td>
<td>4.51</td>
</tr>
<tr>
<td>MAX</td>
<td>12.3</td>
<td>10.1</td>
<td>11.8</td>
<td>21.0</td>
<td>19.2</td>
</tr>
<tr>
<td>MIN</td>
<td>5.30</td>
<td>3.71</td>
<td>8.54</td>
<td>8.80</td>
<td>9.98</td>
</tr>
<tr>
<td>RSD %</td>
<td>29.1</td>
<td>32.3</td>
<td>16.5</td>
<td>28.7</td>
<td>31.1</td>
</tr>
<tr>
<td>Relative Range %</td>
<td>80.1</td>
<td>92.2</td>
<td>31.0</td>
<td>86.5</td>
<td>63.6</td>
</tr>
<tr>
<td>MAX/MIN</td>
<td>2.32</td>
<td>2.72</td>
<td>1.38</td>
<td>2.39</td>
<td>1.92</td>
</tr>
</tbody>
</table>

3.3 Cross-sectional study: Disease state

The distribution of molar APAP-glucuronide/APAP and APAP-sulphate/APAP ratios from 108 patients and 36 control subjects are presented in Figure 2. The frequency of controls has been normalized to 108. Statistical comparison of the two populations showed no significant differences in either glucuronidation or sulphation ratios between seropositive subjects and controls, Table 3. However, both mean conjugation ratios were lower among seropositive subjects than among controls, and these differences approached significance in the case of sulphation (p = 0.055). A larger study might prove these trends to be significant.
Figure 2: Variation in Paracetamol Molar Glucuronidation (A) and Sulphation (B) Ratios Among 108 HIV Positive Patients (■) vs. 36 Controls (□ - Normalized Data)

No statistically significant differences in either glucuronidation or sulphation ratios were observed when patients with AIDS were compared to seropositive, non-AIDS patients and controls, Table 4, p > 0.1. No significant correlation between sulphation ratios and CDC disease stratum was observed, Table 5.
### Table 3: Statistical Summary of Glucuronidation and Sulphation Ratios from 108 HIV Positive Patients and 36 Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>Glucuronidation Ratio</th>
<th>Sulphation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV Pos</td>
<td>HIV Neg</td>
</tr>
<tr>
<td>Sample size</td>
<td>108</td>
<td>36</td>
</tr>
<tr>
<td>Mean</td>
<td>20.9</td>
<td>23.8</td>
</tr>
<tr>
<td>SD</td>
<td>14.9</td>
<td>12.9</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.61</td>
<td>1.46</td>
</tr>
<tr>
<td>Maximum</td>
<td>85.7</td>
<td>60.3</td>
</tr>
<tr>
<td>Median</td>
<td>16.4</td>
<td>21.4</td>
</tr>
</tbody>
</table>

### Table 4: Statistical Summary of Glucuronidation and Sulphation Ratios from 36 Control Subjects and 108 HIV Positive Patients Sorted According to Disease State

<table>
<thead>
<tr>
<th></th>
<th>Glucuronidation Ratio</th>
<th>Sulphation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nonAIDS</td>
<td>AIDS</td>
</tr>
<tr>
<td>Sample Size</td>
<td>67</td>
<td>41</td>
</tr>
<tr>
<td>Mean</td>
<td>21.8</td>
<td>19.5</td>
</tr>
<tr>
<td>SD</td>
<td>16.5</td>
<td>12.0</td>
</tr>
<tr>
<td>Minimum</td>
<td>2.23</td>
<td>0.61</td>
</tr>
<tr>
<td>Maximum</td>
<td>85.7</td>
<td>45.9</td>
</tr>
<tr>
<td>Median</td>
<td>16.6</td>
<td>16.3</td>
</tr>
</tbody>
</table>

### Table 5: Statistical Summary of Sulphation Ratios from 36 Control Subjects and 108 HIV Positive Patients Sorted According to CDC Stratum

<table>
<thead>
<tr>
<th></th>
<th>Sulphation Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Sample size</td>
<td>36</td>
</tr>
<tr>
<td>Mean</td>
<td>13.9</td>
</tr>
<tr>
<td>SD</td>
<td>8.32</td>
</tr>
<tr>
<td>Minimum</td>
<td>4.51</td>
</tr>
<tr>
<td>Maximum</td>
<td>39.2</td>
</tr>
<tr>
<td>Median</td>
<td>11.6</td>
</tr>
</tbody>
</table>
3.4 Cross sectional study: Concomitant medications

The impact of concomitant medication on APAP-glucuronide/APAP and APAP-sulphate/APAP metabolic indices was investigated through regression analyses. The medication - metabolic index pairs whose regression lines had slopes significantly different from zero were further tested for significance using an analysis of variance. This univariate approach was supplemented by a multivariate stepwise regression, which selected, from among all medications, essentially the same subset of significant predictors of metabolic indices. This combined approach led to testing the effect of dapsone, lamivudine, pentamidine and zidovudine on one or both conjugation pathways.

3.4.1 Dapsone The concomitant administration of dapsone appeared to have no significant effect on the glucuronidation of APAP. However, the mean sulphation index was significantly lower among patients taking dapsone than among controls (5.23 vs. 13.9, $p < 0.05$) as well as between patients on dapsone and patients not on the drug (5.23 vs. 11.4, $p = 0.048$).

3.4.2 Lamivudine The concomitant administration of lamivudine appeared to have no significant effect on the glucuronidation of APAP. However, the sulphation ratio among lamivudine users was significantly lower than among controls (8.44 vs. 13.9, $P < 0.05$) as well as patients not on the drug (8.44 vs. 11.9, $p = 0.04$).

3.4.3 Pentamidine Statistical analysis of the data indicated that pentamidine use may be associated with a trend toward increased glucuronidation, median glucuronidation indices = 29.4 {patients receiving pentamidine} vs. 21.4 {controls} vs. 15.8 {patients not receiving pentamidine therapy}, $p = 0.056$. However, the difference in the medians between patients receiving pentamidine and patients who were not, was not significant ($p = 0.12$). Pentamidine appeared to have no significant effect on the sulphation indices.
3.4.4 Zidovudine Zidovudine was examined as having a potential impact because of significant or near-significant non-zero slopes vs. sulphation ($p = 0.05$) and glucuronidation ($p = 0.08$). No differences attributable to zidovudine were seen in glucuronidation and the borderline difference ($p = 0.07$) in sulphation was due to the underlying difference between seropositive patients and controls, mean sulphation indices = 13.9 {controls}, 10.0 {patients receiving zidovudine}, 11.9 {patients not receiving zidovudine}.

3.4.5 Other factors Neither smoking nor caffeine nor alcohol consumption was found to have any significant impact on either conjugation pathway. The coadministration of other glucuronidated substrates was also investigated. Subjects were assigned values indicating total use of such substrates (e.g. patients receiving both oxazepam and zidovudine were assigned 2; patients on no such substrates were assigned 0). The glucuronidation-substrate values were then compared to the glucuronidation indices and no statistically significant correlation was observed ($r = 0.108, p = 0.198$). The effect of oral contraceptives was not analyzed since only one control and one patient were taking them.

3.5 Nutritional status

Nutritional status was assessed in terms of percent difference from ideal body weight (IBW). Regression analysis of % difference from IBW vs. glucuronidation index showed the slope to be significantly different from zero ($r = 0.246, p = 0.006$). The mean of the glucuronidation indices among patients who were underweight by 10% {18.0} was compared to the means of patients with normal weight and those who were overweight by at least 20% {21.1 and 28.2, respectively}. Although a trend towards reduced glucuronidation was observed, it did not reach statistical significance ($p = 0.07$). There was no apparent correlation between nutritional status and sulphation index.
3.6 Longitudinal study:

Repeat determinations of the glucuronidation and sulphation indices were performed with 46 patients: 42 were tested twice and 4 were tested 3 times. Based upon the results from the longitudinal study of control subjects, only changes in the metabolic indices which were greater than ± 3-fold were considered substantive and will thus be referred to as "real" changes.

Real decreases in the glucuronidation index were seen in 8 patients (7.2 ± 4.5 fold decrease) and decreased sulphation indices were observed in 7 patients (5.1 ± 2.2 fold decrease). Six of the 7 patients with reduced sulphation indices also had substantially reduced glucuronidation indices. Increases in conjugation capacity were also observed in 2 patients, one showed 3.7- and 3.1-fold increases in the glucuronidation and sulphation indices, respectively; and the other displayed a 4.1-fold increase in sulphation index.

Four patients were assessed on three occasions. No real changes in the metabolic indices were observed in two such patients. In another such patient, no real change in the glucuronidation index was observed at the second assessment but a 3.4-fold decrease was seen at the third assessment. The fourth patient had a 4.6-fold decrease in the glucuronidation index between the first and second testing and a 3.7-fold increase when tested for the third time.

An approach similar to that used in the analysis of the cross sectional data was used in an effort to explain the observed longitudinal changes. Changes in disease state, medication and body weight were examined.

3.7 Longitudinal study: Disease state

Patients' medical charts were scrutinized and changes in their medical conditions and CDC stratum were tabulated. Disease progression was defined as having occurred in patients who were diagnosed with a new HIV-related or AIDS defining condition between metabolic assessments or whose previously diagnosed condition had become active at the time of the second (or third) test.
HIV-related disease progression between the first and second assessments was observed in 24 of the 46 patients. In all but 8 of these patients, no real changes occurred in either index. Glucuronidation was decreased in 4 patients, 2 of whom also showed decreased sulphation. Sulphation alone was decreased in 2 patients while 1 patient showed increases in both indices. Glucuronidation alone was increased in the eighth patient.

Among the 22 patients who had shown no disease progression, three exhibited a decrease in both metabolic indices, one had increased and two had decreased glucuronidation ratios. The odds ratios of the 2 pathways vs. disease progression were calculated and demonstrated that decreased capacity in either pathway, compared with unchanged or increased capacity, bore no relation to disease progression ($p = 0.72$ and $1.00$ for glucuronidation and sulphation, respectively).

3.8 Longitudinal study: Concomitant medications

3.8.1 Dapsone  Dapsone, seen to significantly decrease the APAP-sulphation index in the cross sectional portion of the study, was in use by only two of the patients tested more than once. These patients ceased taking the drug between the first and second assessments and their APAP-sulphation indices increased 1.4- and 2.9-fold (not deemed to be a "real" change by the study criteria). There was no significant difference in the two measurements ($p = 0.13$), although such a small sample can neither confirm nor contradict dapsone's potential effect on the sulphation of APAP.

3.8.2 Lamivudine  Twenty-two patients began lamivudine therapy between the first and second metabolic assessments. A real decrease in the sulphation index was seen in six patients, an increase in the index occurred in one, and 15 displayed no real change. The patients on lamivudine had a lower mean APAP-sulphation index than when they were not on the drug ($8.23$ vs. $11.6$) but this difference did not reach significance, $p = 0.08$. This result is inconsistent with the data from the cross sectional portion of the study. No real changes were
seen in either the APAP-glucuronidation or APAP-sulphation indices in the 7 patients who were on lamivudine for both tests. The fifteen patients who were not on lamivudine at the time of the first test but went on it during the interval showed a significant decrease in glucuronidation index (-7.2, p = 0.04) and in sulphation index (-5.9, p < 0.01).

3.8.3 Pentamidine Eight patients either began or ceased taking pentamidine between the first and second metabolic assessments. The mean APAP-glucuronidation index of those patients when not taking pentamidine was 23.8 while when taking the drug, the mean index was 29.3. The observed difference did not prove to be significant, p = 0.3 and pentamidine appears to have no effect on APAP-glucuronidation.

3.8.4 Zidovudine Seventeen patients either began or ceased taking zidovudine between the first and second metabolic assessments. Two of the patients displayed a real increase in their APAP-glucuronidation index when they were on the drug relative to when they were not while zidovudine administration had the opposite effect in 1 patient. The mean APAP-glucuronidation index of the patients when they were not taking zidovudine was 15.8 compared with a mean index of 24.4 while they were on the drug. The observed difference was significant, p = 0.03. The pharmacological and clinical significance of this difference is not clear considering that only 3 of 17 patients had real changes (>3-fold) and that all of the changes were not in the same direction. The thirteen patients who were taking zidovudine during both assessments showed a significant decrease in glucuronidation index (-8.9, p = 0.01) and sulphation index (-4.4, p = 0.03).

3.9 Longitudinal study: Nutritional status

There was very little longitudinal data with which to assess the effect of nutritional status on the APAP-glucuronidation and APAP-sulphation indices. Only two patients had any substantial weight loss (>10%). One showed a decrease in her APAP-glucuronidation index and the other showed an increase
while no changes were observed in the APAP-sulphation indices. These two patients were assessed a third time, at which point they had regained the weight and their APAP-glucuronidation indices returned to their initial values.

4. Discussion

This study was designed to determine the relative glucuronidation and sulphation activities in HIV positive patients and patients with AIDS. The initial selection criteria was a CD4+ cell count of 150 - 250 mm$^{-3}$ (or <20% of lymphocytes) at which point HIV disease progression is likely to occur. Many of the individuals would be likely to develop AIDS defining events and are thus a population in which the activities of UDP-glucuronosyltransferase (UDP-GT) and sulphotransferase (ST) may be affected by the administration of multiple medications or by HIV disease activity such as the onset of new opportunistic infections or tumours. Some patients, selected to participate on this basis, had undergone important changes in CD4+ cell count by the time they were recruited and participated, hence the inclusion of some subjects outside the target range of the protocol.

The method chosen to assess the activities of UDP-GT and ST was the glucuronidation and sulphation of APAP. The approach was based on the work of Bock et al. [ibid.] which used a 1 g dose of APAP and a 0-8 h cumulative urine collection. The HPLC method used in this study permitted the dose to be lowered to 500 mg, thereby reducing the drug burden of the patients.

The 0-8 h cumulative urine collection was replaced by a 4-h spot urine sample. Validation of the method in healthy controls demonstrated that an APAP-glucuronidation index {molar concentration ratio APAP-glucuronide/APAP} and an APAP-sulphation index {molar concentration ratio APAP-sulphate/APAP} could be produced using the data from the HPLC analysis of the parent and metabolite concentrations in the 4-h urine sample. The method validation study also demonstrated that the indices determined in 4-h urine samples did not significantly
differ from the same indices determined from the HPLC analysis of cumulative 0-8 h urine samples. These results are consistent with the previous data of Miller et al. [1976] which suggested that the conjugated to free APAP ratio should not significantly differ between samples collected hourly between 3- and 7-h post-dose from that found in an 8-h cumulative sample.

This study contained both cross sectional and longitudinal components. In order to establish some criteria to define real inter-day changes in UDP-GT and ST activities in the patient population, 5 healthy volunteers were evaluated on 3-8 nonconsecutive days. In these subjects, the calculated APAP-glucuronidation and APAP-sulphation indices varied ± 2.8-fold. Therefore, in the longitudinal study, only changes in the calculated indices which were > 3-fold were considered real.

The results from the cross sectional study of APAP glucuronidation and sulphation in the seropositive and control populations are presented in Figure 2. The histograms show very similar unimodal distributions for the two populations with only slight differences in mode and rather more outliers among the seropositive subjects. The mean APAP-glucuronidation and APAP-sulphation indices for the controls, seropositive non-AIDS and AIDS groups ranged from 19.5 - 23.8 and 9.8 - 11.7, respectively, Table 4. These ratios are slightly higher and lower, respectively, than those previously reported by Bock et al. [1987] who measured ratios of 18 for glucuronidation and 12 for sulphation among non-smoking controls.

Statistical analysis of the cross sectional data revealed no significant differences among the 3 groups relative to the APAP-glucuronidation and APAP-sulphation indices, although there may be a trend toward a lower APAP-sulphation index among seropositive subjects (11.0) vs. controls (13.9), p = 0.055, Table 3. The distribution of the APAP-sulphation indices was further analyzed by separating the seropositive patients according to their CDC status, Table 5. No significant differences were seen between any of the resulting 4 groups.
These observations differ from the results recently presented by Esteban et al. [1997] which involved 32 controls, 9 asymptomatic HIV positive patients and 19 AIDS patients. The data from that study indicated a significant decrease in the excretion of APAP-glucuronide and an increase in APAP-sulphate excretion in patients with AIDS compared to asymptomatic HIV positive patients or controls. The study, based on total excretion of the metabolites over 24 hours and not on metabolite/parent ratios, involved far fewer subjects and had no longitudinal phase.

The data from the longitudinal study also differs from the observations of Esteban et al. [ibid.] and is consistent with the view that disease state does not affect the glucuronidation or sulphation of APAP. In this study, disease progression was defined as having occurred in patients who were diagnosed with a new HIV-related or AIDS defining condition between metabolic assessments or whose previously diagnosed condition had become active at the time of the second (or third) test. Using these criteria, HIV-related disease progression was observed in 24 of 46 patients. Real increases and decreases (> 3-fold changes) in the glucuronidation and sulphation indices were observed in both the progressing and non-progressing cohorts. The odds ratios relative to real changes in one or both of the metabolic indices vs. disease progression were calculated and demonstrated that decreased capacity in either pathway, compared with unchanged or increased capacity, bore no relation to disease progression (p = 0.72 and 1.00 for glucuronidation and sulphation, respectively).

Previous investigations have identified a number of environmental factors which affect APAP metabolism. These include: tobacco use [Bock et al. 1994; Mucklow et al. 1980], gender [ibid.], oral contraceptive use [ibid.], race [Mucklow et al. ibid.] and alcohol consumption [ibid.]. In this study, neither smoking nor consumption of caffeine or alcohol was found to have any significant impact on either conjugation pathway.

Concomitant drug administration has also been shown to have an effect on APAP metabolism and phenytoin and rifampicin have been shown to induce APAP-glucuronidation [Bock et al. 1987]. The data from the cross sectional portion of this
study suggests that dapsone may depress APAP-sulphation although the results from the longitudinal section failed to confirm this. Since dapsone is glucuronidated, coadministration of this drug might have been expected to competitively decrease APAP glucuronidation, although no other glucuronidated concomitant medication, nor the sum of such medications, had such an effect. Why dapsone should interfere with APAP-sulphation is not clear nor has this potential interaction been reported elsewhere.

The literature offers conflicting views on the potential effect of zidovudine on APAP glucuronidation. Recent reports suggest that coadministration of zidovudine might potentiate APAP-induced hepatotoxicity [Shriner & Goetz, 1992]. However, Burger et al. [1994] have shown neither drug to affect the pharmacokinetics of the other. The data from the cross sectional segment of this study is consistent with the latter finding as no significant difference was detected in either APAP-glucuronidation or APAP-sulphation indices between patients taking zidovudine and those not taking it.

In the longitudinal portion of the study, zidovudine was associated with a statistically significant increase in the APAP-glucuronidation index as observed in 17 patients who were tested both while taking the drug and not taking it. However, the absolute change in this index was only considered real \( \text{i.e.} > 3\text{-fold} \) in 3 of those patients and it was a positive change for 2 and a negative change for the other. Thus, it is unclear if the statistically derived significance has any clinical or pharmacological relevance. Further longitudinal studies will be required to answer this question.

Protein-calorie malnutrition was identified as another factor which may reduce APAP glucuronidation and sulphation in rats [Price et al. 1987]. In this study, nutritional status was assessed on the basis of percent difference from ideal body weight (IBW). The mean APAP-glucuronidation index of patients who were under their IBW by at least 10% was lower than the mean indices of patients whose weight was normal or those who were overweight by 20% or more, and this trend approached significance \( (p = 0.07) \). However, % difference from IBW is far from ideal as a
measure of a patient’s nutritional status. Lean body mass measurements would be required to determine if AIDS-related wasting or other defects in intermediate metabolism were present. Studies designed to address this issues are currently in progress.

5. Conclusion

In this study, the presence of HIV infection or the progression of this infection to AIDS has not been shown to alter the ability of a patient to transform APAP into its glucuronide and sulphate metabolites relative to controls. Environmental factors and concomitant administration of other therapeutic agents have also been shown to have no significant effect on these metabolic pathways with the possible exception of dapsone and lamivudine which were found to depress APAP-sulphation and zidovudine which may increase APAP-glucuronidation. The study has also demonstrated that a 4-hour spot urine sample and the construction of metabolic indices are useful tools in this complex patient population.

6. References


CHAPTER V: Drug metabolism in HIV-positive patients and patients with AIDS: the genotype and expressed phenotype of the microsomal enzyme cytochrome P450 2D6

This paper, by W.M. O'Neil, B.M. Gilfix, N. Markoglu, A. Di Girolamo, C.M. Tsoukas and I.W. Wainer, was submitted to Pharmacogenetics in March, 1998.

The contribution of the co-authors was as follows: Dr. Brian Gilfix performed the genotype determinations on the DNA from the patients and reviewed and corrected the manuscript. Ms. Nektaria Markoglu refined and validated the HPLC assay and performed all of the urine analyses for dextromethorphan and its metabolites. In addition she extracted about half of the urine samples, preparatory to HPLC analysis and condensed all the HPLC data into spreadsheets. Ms. Antonietta Di Girolamo provided expertise in the organization of the clinical data, helped greatly in the perusal of patients' charts for the collection of such data and recruited one quarter of the patients into the study. In addition, she prepared the original draft of a manuscript upon which this manuscript is based. Dr. Christos Tsoukas provided guidance in the design of the experiments, facilitated access to the patient population and reviewed and corrected the manuscript. Dr. Irving Wainer was instrumental in the formulation of the research plan and supervised all aspects of its execution. He reviewed and revised the manuscript. William O'Neil formulated the research plan with Dr. Wainer, drafted the protocol, obtained approval for its execution from the hospital Ethics Committee, designed and prepared the consent forms, patient questionnaires, patient instruction sheets and, with the aid of Ms. Di Girolamo, the case report forms. He recruited three quarters of the patients into the study, processed all patient samples for storage and extracted about half of the urine samples preparatory to HPLC analysis. He prepared the manuscript, based on that of Ms. Di Girolamo and performed revisions under the direction of Drs. Wainer, Gilfix and Tsoukas.
The paper describes the expression of the CYP2D6 pathway, like NAT2 a polymorphic pathway. As seen for NAT2, discrepancies between genotype and phenotype were observed. There had been nothing published previously to suggest such a phenomenon. The genotype was distributed as would be expected for a Caucasian population, 59:2 extensive:poor. On its own the phenotypic data also had a distribution entirely consistent with that seen in similar populations, 103:5 extensive metabolizer (EM):poor metabolizer (PM). However, two of the EM genotypes were PM phenotypes. The fifth PM phenotype's genotype was unknown.

Several of the patients were receiving treatment with agents known to inhibit CYP2D6. They were not among the two discrepant patients (EM/PM's). Furthermore, four patients had metabolic quotients superior to those inhibited patients, indicating diminished, though not PM, CYP2D6 capacity. Inspection of the frequency distribution of CYP2D6 activity revealed a "peak" which included most of the patients on CYP2D6 inhibitors, but not exclusively such patients.

Probit analysis of the metabolic quotient distribution only among EM's, but excluding those receiving CYP2D6 inhibitors, yielded a secondary antimode which separated the population into two groups labelled EM and slow EM. The slow EM pheno-subtype was found to be significantly associated with the presence of an active illness. A previous diagnosis of an AIDS defining illness or progression to AIDS was not associated with the slow EM pheno-subtype. Indeed changes from one pheno-subtype to the other, seen in the longitudinal data, bore no relationship to disease progression or activity. Thus the longitudinal data failed to corroborate the cross sectional data.

This last paper furthers the story of how drug metabolism varies within an HIV positive population and provides some insight as to why. Clearly it does not complete the story. The conclusions are that genotype and phenotype are each inadequate on their own to establish meaningful insight into a patient's metabolic status, as was seen with NAT2. Disease activity seems to affect CYP2D6 expression but processes more fundamental to the HIV infection itself may be involved.
Drug metabolism in HIV-positive patients and patients with AIDS: the genotype and expressed phenotype of the microsomal enzyme cytochrome P450 2D6

William M. O'Neil, Brian M. Gilfix, Nektaria Markoglou, Antonietta Di Girolamo, Christos M. Tsoukas and Irving W. Wainer

Abstract

Objective: To examine the distribution of the cytochrome P450 (CYP) CYP2D6 phenotype and its relation to genotype, concomitant medication and disease state in HIV-positive patients. Design: A cross sectional study with a longitudinal component compared individual genotypes for CYP2D6 to the expressed CYP2D6 phenotype. Methods: Sixty-one, predominately male Caucasian, HIV-positive patients were recruited and CYP2D6 genotypes (extensive metabolizer (EM) or poor metabolizer (PM)) determined by PCR-based amplification followed by restriction fragment length analysis. The patients were also phenotyped using dextromethorphan (DM) to determine their respective expressed enzyme activity and assigned either a CYP2D6 EM or PM phenotype. Complete medical and treatment histories were compiled. An additional 47 patients were studied but only phenotypically. A total of 44 patients were tested longitudinally. Results: Fifty-nine patients (97%) possessed an EM genotype, consistent with previously observed distributions in demographically similar populations. In healthy seronegative populations, genotype and phenotype have been shown to be essentially interchangeable measures of CYP2D6 activity. In this cohort, 2 of the 59 patients with an EM genotype expressed a PM phenotype. An additional 4 patients were less extensive DM metabolizers than any of the patients receiving medication known to inhibit CYP2D6 in vitro. The shift toward the PM phenotype from an EM genotype seemed to be associated with the presence of active illness. Conclusion: Changes in expressed CYP2D6 activity occur within HIV-positive patients with movement toward PM phenotypes from EM genotypes. Active disease alone cannot explain the shift. Something underlying HIV infection itself may be the cause.
Introduction

Cytochromes P450 (CYP's) comprise a key class of metabolizing enzymes and are responsible for the majority of oxidative drug transformations [Gonzalez & Idle, 1994; Daly et al. 1993]. CYP's exhibit a large degree of interindividual variability in their levels of expression and these variations have been related to interpatient differences in drug efficacy and toxicity [Gonzalez & Idle, ibid.].

In most cases, interindividual differences in the activity of CYP isoforms are quantitative as enzymatic activity appears unimodally distributed. Some CYP's are genetically polymorphic and their activity falls into two clearly defined and qualitatively different populations: individuals whose rate and extent of metabolism is lower ("poor" metabolizers, PM) and those who have a faster or more extensive metabolism ("extensive" metabolizers, EM).

The genetic bases for the polymorphisms of CYP2D6, CYP2C18 and CYP2C9 have been identified. For enzymes whose genes have not been identified, the determination of metabolic phenotype (the measure of the actual enzyme activity) remains the only way to assess metabolic differences. This approach utilizes the identification and quantification of specific metabolite patterns produced by test compounds or "probe drugs" [Gonzalez & Idle, ibid.]. Direct correlations have been established between metabolic genotypes and probe drug phenotypes [Kaufmann et al. 1996; Chen et al. 1996].

While genotype appears to govern the constitutive expression of enzymatic activity, changes in the relative levels and activities of metabolizing enzymes can be produced by drug interactions [Correia, 1995], environmental factors (e.g. smoking, alcohol intake, etc.) [Correia, ibid.] and clinical conditions such as disease progression [Correia, ibid.] or malnutrition [Parke & Ioannides, 1981]. Thus a healthy subject's reaction to a drug may differ from that seen when the person's health has degenerated. This phenomenon has been demonstrated in AIDS patients for the activity of the enzyme N-acetyltransferase 2 (NAT2). In two studies, disease progression altered the observed phenotype in patients with NAT2 EM genotypes from phenotypically EM to phenotypically PM [Lee et al. 1993; O'Neil et al. 1997].
The effect of disease progression on the activity of CYP's in HIV-positive patients is unknown. We report here the first studies into this effect using the hepatic microsomal enzyme CYP2D6 as the marker enzyme. CYP2D6 was chosen for the following reasons:

1) The genetics of CYP2D6 have been extensively studied and individual genotypes can be rapidly determined [Eichelbaum et al. 1979]. Humans can be divided into EM and PM genotypes with a population-based distribution which can range from only 7 PM's out of 695 Chinese subjects (1.01%) [Bertilsson et al. 1992] to a PM frequency of 7% in Caucasian populations [Bertilsson et al. ibid.; Schmid et al. 1985; Brøsen & Gram, 1989].

2) The expressed activity of CYP2D6 can be measured by determining the extent of the O-demethylation of the probe drug dextromethorphan (DM). CYP2D6 probe drug phenotypes correlate with genotype [Chen et al. 1996; Jones et al. 1996; Ducharme et al. 1996; Bock et al. 1994] and large population studies and clinical protocols [Bertilsson et al. 1992; Jacqz et al. 1988; Relling et al. 1991] have used DM metabolism as the determinant of PM and EM CYP2D6 activity.

3) The metabolic phenotypes determined using DM metabolism have been shown to be reproducible over time in healthy populations: i.e. PM's remained PM's and EM's remained EM's [Ducharme et al. 1996]. DM is metabolized by at least 2 pathways: CYP2D6 mediated O-demethylation and N-demethylation, at least partially mediated by CYP3A. However, the coadministration of CYP3A inhibitors with DM has shown O-demethylation to be unaffected [Ducharme et al. ibid.]. The 2 pathways are therefore independent.

4) CYP2D6 mediates the biotransformation of several classes of drugs that are of major therapeutic value including cardiovascular drugs [Eichelbaum & Gross, 1990; Meyer et al. 1990], psychotropic agents [Dahl et al. 1993; Brøsen et al. 1991; Llerena et al. 1992], codeine [Yue et al. 1989], fluoxetine [Chen et al. 1996; Stevens & Wrighton, 1993] and ritonavir [Kumar et al. 1996]. In addition, individuals who have a CYP2D6 PM genotype (or phenotype) are at increased risk for developing drug-related toxicities when drugs metabolized by this pathway are administered [Hamelin et al. 1996]. Thus, a disease induced shift toward a PM phenotype could produce previously unobserved toxicities.
In this study, 108 HIV-positive patients were probe-drug phenotyped with DM. The \textit{CYP2D6} genotype was determined in 61 of the patients by PCR-based amplification followed by restriction fragment length analysis. Forty-four patients were phenotyped more than once. Complete medical and treatment histories were also obtained. The results suggest that the relationship between phenotype and genotype can be altered within an HIV-positive population with \textit{EM} genotypes expressed as PM phenotypes.

\textbf{Patients and methods}

\textbf{Study population}

Patients were recruited into the study from among those actively followed at the Montreal General Hospital (MGH) Immunodeficiency Treatment Centre. Approval from the MGH Ethics Committee had been obtained prior to the start of the study. At the time of enrolment, a detailed patient HIV history, concomitant medications, and clinical status were obtained. Current drug therapy was continued. Written informed consent was obtained from each patient before initiation of the phenotyping and genotyping studies.

\textbf{CYP2D6 phenotyping}

Each participant received a single 30 mg oral dose of dextromethorphan (10 mL of Robitussin DM\textsuperscript{\textregistered}, donated by Whitehall-Robins Inc., Mississauga, ON, Canada). Pre-dose and 4-hour post-dose spot urine samples were collected and frozen at -20°C until analyzed.

The urinary molar concentrations of dextromethorphan (DM) and its O-demethylated metabolite, dextrorphan (DR), were determined using an HPLC method described by Park \textit{et al.} [1984] modified by the use of a C\textsubscript{18} solid-phase extraction cartridge. DM/DR molar ratios were used to assign phenotypes. In cases where the DM concentration was below the limit of quantitation, a value of 0.08 μM, equal to 80% of the limit of quantitation was arbitrarily inserted.

\textbf{CYP2D6 genotyping}

A single 7 mL venous blood sample was collected into a tube containing ethylenediaminetetraacetic acid (EDTA) and stored at -20°C until the DNA was extracted. All restriction buffers, bovine serum albumin (BSA), and restriction enzymes were from
New England Biolabs Ltd (Mississauga, ON, Canada). All primers were synthesized by Sheldon Biotechnology Centre (Montreal QC, Canada).

DNA was isolated from lymphocytes or whole blood using a commercial kit (Puregene DNA Isolation kit, Gentra Systems, Minneapolis, MN, USA). This extraction method allowed the introduction of a heating step (60°C for 1 h) during the cell lysis stage providing for the inactivation of any HIV. The DNA isolated from 1 x 10⁶ lymphocytes or 300 µL whole blood was dissolved in 100 µL of the supplied DNA resuspension buffer.

The identification of the \(CYP2D6(A)\) (\(del\) A2637; \(CYP2D6*3\)) and \(CYP2D6(B)\) (\(G1934A;\) \(CYP2D6*4\)) mutations was performed by restriction genotyping using a modification of the method of Douglas et al. [1994]. Reactions were carried out in a volume of 50 µL containing 5 µL (~0.3 µg) of DNA; deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate and deoxyguanosine triphosphate, each at 0.2 mM; 12.5 pmol of primer 2D6-1827 (5' CGCCTTCGCCAACC-ACTCCG 3'); 12.5 pmol of primer 2D6-2662 (5' GGCTGGGCTGGGTCCCAGGTCA-TAC 3'); and 5 µL of 10 x PCR reaction buffer (100 mM Tris HCl, pH 9.0, 500 mM KCl, 15 mM MgCl₂, 2 mg/mL gelatin, 1% Triton X100) (Vector Biosystems, Toronto, ON, Canada).

The DNA in the reaction mixture was first denatured for 10 min at 96°C and then cooled to 4°C. One unit of Taq polymerase (Vector Biosystems) was then added to each sample. Each sample was reheated for 2 min at 96°C and then subjected to 30 cycles in a thermal cycler with each cycle consisting of a 10 sec denaturation at 96°C, 30 sec annealing at 60°C and 1 min extension at 65°C.

To identify the \(CYP2D6(A)\) mutation, 10 µL of the amplified material was mixed with the following: 1.5 µL of NEBuffer R; 0.2 µL 10 mg/mL BSA; 0.33 µL of Taq I (6 units/mL); and 2.97 µL of water. The restriction mixture was overlaid with mineral oil and incubated overnight at 65°C.

To identify the \(CYP2D6(B)\) mutation, 10 µL of the amplified material was mixed with the following: 1.5 µL of NEBuffer 2; 0.2 µL 10 mg/mL BSA; 1 µL of BstN I (10
units/mL); and 2.3 μL of water. The restriction mixture was overlaid with mineral oil and incubated overnight at 60°C.

The identification of the CYP2D6(D) (CYP2D6 deleted; CYP2D6*5) was performed by a "long PCR" technique using the Expand™ Long Template PCR System (Boehringer Mannheim Canada, Laval, QC, Canada) [Daly & Steward, 1995]. Reactions were carried as described for the identification of the CYP2D6(A) and CYP2D6(B) mutations except the DNA and nucleotides were incubated with 12.5 pmol of primer CYP-13 (5' ACCGGGCACCTGTACTCCTCA 3'); 12.5 pmol of primer CYP-24 (5' GCATGAGCTAAGGCACCCAGAC 3'); 5 μL of 10 x Expand™ buffer 1; and 0.75 μL (2.5 units) of Expand™ Long Template enzyme mixture.

Each sample was heated for 1 min at 93°C and then subjected to 30 cycles in a thermal cycler with each cycle consisting of a 1 min denaturation at 93°C, 2 min annealing at 65°C and 10 min extension at 68°C. The heating and cooling rates for all steps were 1°C/sec except for cooling from 93°C to 65°C where the rate was 0.5°C/sec.

**Gel electrophoresis**

The restriction products were analyzed by electrophoresis of 10 μL of the reaction mixture on a 10% polyacrylamide gel containing TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) for 2.3 h at 70 V. The gel was then stained for 45 min in 0.5 μg/mL ethidium bromide, photographed under UV light, and the banding profile identified on the basis of molecular weight standards.

The products from the "long PCR" reaction were analyzed by electrophoresis of 10 μL of the reaction mixture on a 0.8% agarose gel containing TPE buffer (80 mM Tris-phosphate, 2 mM EDTA) and 0.156 μg/mL ethidium bromide for 1 h at 68 V. The gel was then photographed and the banding profile identified as above.

**Statistical analysis**

Data are presented as mean ± SD. Odds ratios were calculated by use of Fisher's exact test and the 95% confidence interval with the approximation of Woolf (Graphpad InStat version 2.04).
Results

Patient characteristics:

One hundred eight patients were recruited into the study. Forty-four of those subjects were phenotyped on more than one occasion (mean time between phenotypes: 12.8 months). Blood samples were extracted and CYP2D6 genotype determined for 61 of the patients, 35 of whom were phenotyped more than once. The age of the 108 unrelated, HIV-positive patients ranged from 26-74 (43 ± 10 y), 90% were male and 87% were Caucasians. The CD4+ cell count of the patients ranged from 2 mm$^{-3}$ to 1029 mm$^{-3}$ (219 ± 140 mm$^{-3}$), 42 (39%) had AIDS and 20 (18%) had active disease at the time of initial testing. Of the 61 patients who were genotyped, 20 (33%) had AIDS including 15 (24%) who had active disease at the time of initial testing. The CD4+ cell count of genotyped patients ranged from 2 mm$^{-3}$ to 1029 mm$^{-3}$ (mean 231 ± 154 mm$^{-3}$). The classification of AIDS was based on the existence of a current or previous diagnosis of an AIDS-defining illness as defined by the Centers for Disease Control [1992], but irrespective of CD4+ cell count. Active illness refers to current diagnoses, AIDS-defining or other.

Renal profiles and liver function tests were within normal range or ≤ grade 1 elevation for all but six patients one of whom demonstrated an elevated bilirubin (direct and indirect) three times the upper limit of normal. Alkaline phosphatase and liver transaminase for this patient were within normal limits. Two patients had grade 2 elevations of bilirubin and two had elevated (grade 2) transaminase. One patient had grade 3 elevation of liver transaminase with normal bilirubin.

Genotypes:

Our use of restriction genotyping [Douglas et al. 1994] and "long PCR" [Daly & Steward, 1995] allowed the identification of the following three PM-associated alleles (along with their estimated allele frequency in the Caucasian population) [Sachse et al. 1997]: CYP2D6(A) (0.020), CYP2D6(B) (0.207) and CYP2D6(D) (0.0195). (These alleles are known in the newer nomenclature as CYP2D6*3, *4 and *5, respectively [Daly et al. 1996]). Other PM-associated alleles are known, but are relatively infrequent in the population, for example, CYP2D6*6 (0.0093) and CYP2D6*15 (0.0008) [Sachse et al.
ibid.; Daly et al. ibid.). Genotyping alone for the CYP2D6(A), CYP2D6(B) and CYP2D6(D) mutations would allow for the identification of 95% of the PM-associated alleles, 88% of genotypes where both alleles were of the slow type, and 98% of genotypes where one allele was EM type and the other PM type based on a recent study [Sachse et al. ibid.]. Thus, as the PM-associated alleles act recessively, genotyping for the 3 mutations

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CYP2D6 Genotype</th>
<th>[DM]/[DR]</th>
<th>Phenotype</th>
<th>Patient No.</th>
<th>CYP2D6 Genotype</th>
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*PM phenotype despite EM genotype
mentioned above would allow the correct classification in the large majority of cases into extensive metabolizer (EM) and poor metabolizer (PM) genotypes.

Fifty-nine patients (97%) possessed an extensive CYP2D6 metabolizer (EM) genotype, while two patients (3%) possessed a poor CYP2D6 metabolizer (PM) genotype, Table 1. These results correlate with previously observed data in a predominantly male Caucasian population [Jones et al. 1996; Ducharme et al. 1996; Bock et al. 1994].

**Cross sectional study**

The frequency distribution of [DM]/[DR] ratios among the 108 patients is shown in Figure 1, broken down according to genotype. The histogram suggests that the distribution may be trimodal but probit analysis of the data yielded a single antimode at 0.6, consistent with published values for populations of similar demographics [Schmid et al. 1985; Ducharme et al. 1996]. The distribution of phenotype according to that antimode is 103:5 EM:PM (95%:5%) which would have been expected except that two genotypic EM's were phenotypically PM.

Figure 2 is a histogram showing only the patients of known genotype and indicates those who were receiving medications which, in vitro, have been shown to inhibit CYP2D6. Thus is it evident that the discrepancies between genotype and phenotype observed in the two genotypically EM patients who displayed a PM phenotype, cannot be explained by metabolic drug interactions.

The EM's receiving medication inhibitory to CYP2D6 were clustered around a "peak" at \( \log([\text{DM}]/[\text{DR}]) = -1.2 \) (Figure 2). This suggests that CYP2D6 inhibitors can reduce the observed enzyme activity but not cause a phenotypic change from EM to PM. Even patients on more than one CYP2D6 inhibitor failed to have their EM genotype perturbed to the point that they could be classified as PM phenotypes, Table 2.

When patients receiving CYP2D6 inhibitors were removed from consideration, the peak at -1.2 remained in the histogram (Figure 2) as did the suggestion of two metabolic populations. In order to examine what might distinguish the secondary population of "slow" EM's from normal EM's, a probit analysis was performed on the EM's alone, minus those taking CYP2D6 inhibitors. This yielded an antimode of -1.5 \( \{\log([\text{DM}]/[\text{DR}])\} \)
Figure 1: Frequency distribution of CYP2D6 phenotype among 108 HIV-positive patients. □ Patients with an EM genotype; ■ Patients with a PM genotype; ▲ Patients of unknown genotype.

Figure 2: Frequency distribution of CYP2D6 phenotype among 61 HIV-positive patients of known genotype. □ Patients with an EM genotype; ■ Patients with an EM genotype receiving medication known to inhibit CYP2D6 in vitro (e.g. cimetidine, fluoxetine, methadone, quinine); ▲ Patients with a PM genotype.
Table 2: Disease and medication characteristics of 61 HIV-positive patients sorted by [DM]/[DR] ratio

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\(a\) EM = extensive metabolizer; PM = poor metabolizer; lower case = heterozygote

\(b\) Classifications are according to CDC strata where A signifies asymptomatic, B signifies a current or previous diagnosis of an HIV-related, but not AIDS-defining illness (e.g. oral candidiasis), C signifies a current or previous AIDS-defining illness, (e.g. PCP) and * indicates an illness active at the time of phenotyping.

\(c\) Medications known to inhibit CYP2D6 in vitro, where q = quinine, c = cimetidine, f = fluoxetine, m = methadone

\(d\) Patients with a PM phenotype despite an EM genotype
equivalent to a $[\text{DM}]/[\text{DR}]$ ratio of 0.03. This supported the existence of two subpopulations: normal EM's and "slow" EM's. This distinction, EM vs. "slow" EM, was then used to explore how disease progression might impact on CYP2D6 expression.

Of 108 patients in the cross sectional study, 8 were receiving CYP2D6 inhibitors, two had a $PM$ genotype and one, of unknown genotype, expressed a PM phenotype. The remaining patients comprised a cohort of 97 experimental subjects. Of those, 38 had AIDS, 26 whose $[\text{DM}]/[\text{DR}]$ ratio was less than 0.03 (EM's) and 12 whose $[\text{DM}]/[\text{DR}]$ ratio was greater than 0.03 ("slow" EM's). This compared with 59 patients who did not have AIDS (12:47 "slow" EM:EM; Tables 2, 3). The odds ratio associating a $[\text{DM}]/[\text{DR}]$ ratio $> 0.03$ with AIDS compared with a $[\text{DM}]/[\text{DR}]$ ratio $< 0.03$ with AIDS was 1.8 (95% confidence interval 0.7 - 4.6; not significant, Table 3). Similarly, among the 54 $EM$ genotypes not receiving CYP2D6 inhibitors, the odds ratio was 3.2 (95% confidence interval 0.9 - 11.6; not significant, Table 3).

Of the 97 patients in this cohort, 28 were suffering from some opportunistic infection or tumour when phenotyped. Of those 28 patients, 13 were on the "slow" side of the secondary antimode and 15 were "normal" EM's. Among the 69 patients without an active illness the distribution was 11:58 "slow" EM:EM. Based upon this distribution, the effect of active illness on CYP2D6 activity was investigated through the calculation of the odds ratio associating an active infection or tumour with a $[\text{DM}]/[\text{DR}]$ ratio $> 0.03$ compared with no active disease. The result was a ratio of 4.6 (95% confidence interval 1.7 - 12.2; significant, Table 3) indicating a probable causal relationship between active disease and a "slow" EM phenotype.

The nature of the active illness was not seen to affect the relationship. The percentage of AIDS-defining illnesses active among EM subjects was similar to that among "slow" EM subjects (50% vs. 40%) and the only remarkable difference in disease type was that four "slow" EM patients had active $pneumocystis carinii$ pneumonia (PCP) while none of the EM patients did. Liver function $\text{per se}$ did not seem to be a factor although one of the discrepant PM's (No. 7184) had grade 3 hyperbilirubinaemia. Two patients with a grade 2 elevation of bilirubin included an EM and a "slow" EM phenotype. Three patients with
Table 3: Odds ratios comparing pheno-subtype with A) AIDS status among all patients, A) AIDS status among all patients with an EM genotype and C) presence of active illness among all patients

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Odds Ratio = 1.8 (95% CI: 0.7 - 4.6, ns)

### B

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Odds Ratio = 3.2 (95% CI: 0.9 - 11.6, ns)

### C

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<td>Patients with active illness</td>
<td>13</td>
<td>15</td>
<td>28</td>
</tr>
<tr>
<td>Patients with no active illness</td>
<td>11</td>
<td>58</td>
<td>69</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>73</td>
<td>97</td>
</tr>
</tbody>
</table>

Odds Ratio = 4.6 (95% CI: 1.7 - 12.2)

* except those with a PM genotype, those on CYP2D6 inhibitors, and the patient of unknown genotype who had a PM phenotype
* except those on CYP2D6 inhibitors
* AIDS designation based on diagnosis irrespective of CD4+ count

Fisher's Exact Test for association between rows and columns, 2-sided P value: 0.24 (ns), 0.096 (ns), 0.0035 (considered very significant)
elevated transaminase comprised two EM's (including the patient with grade 3 elevation) and a "slow" EM.

**Longitudinal study**

Forty-four patients were phenotyped more than once and three were phenotyped three times. All but two were Caucasian and 39 were male. Mean age was 43.3 and 44.4 y on the first and second occasion, respectively. Mean CD4+ cell count was 189 mm$^{-3}$ and 232 mm$^{-3}$ on the first and second occasion, respectively. Genotypes were known for 35 of those patients, all but one being EM. When tested the second (or third) time all patients' phenotypes matched their genotypes. Therefore, the two patients who were phenotypically PM despite an EM genotype (patients No. 6151 and 7184), reverted to an EM phenotype upon retesting.

Besides these changes, from PM to "slow" EM (No. 7184) and from PM to EM (No. 6151), there was one patient of unknown genotype who went from an EM to a PM phenotype. Fourteen patients showed movement within their EM classification: six patients went from EM to "slow" EM and eight went from "slow" EM to EM. There was no association between disease progression, defined as having occurred in patients who were diagnosed with a new HIV-related or AIDS defining condition between the first and second metabolic assessments, and this movement from one side of the 0.03 antimode to the other. Likewise, there was no association between pheno-subtype and the presence of an illness active at the time of phenotyping. For example, of the six patients whose classification changed from EM to "slow" EM, two exhibited disease progression (four didn't) and the presence of diseases in an active state was largely unchanged. Even the patient whose phenotype changed from EM (metabolic ratio = 0.020) to PM (metabolic ratio = 2.43) remained asymptomatic. Similarly, of the eight patients who switched the other way ("slow" EM to EM) four progressed and four didn't and none displayed any change in active disease status. The apparent relationship between active disease and CYP2D6 activity, seen in the cross sectional segment of the study, was not found when the same test was applied to the data collected from the 40 patients who, at their second phenotyping, were not receiving CYP2D6 inhibitors.
Discussion

In the 61 genotyped patients included in this study, the distribution of EM and PM genotypes was consistent with the distribution found in healthy, seronegative populations. The data suggest that conversion to seropositive status and progression to AIDS has no relationship to metabolic genotype. This is consistent with previous studies of the distribution of NAT2 genotypes, in which no differences in genotypic distribution were found in matched cohorts of healthy, seronegative populations and AIDS patients [Lee et al. 1993; Delomémie et al. 1994].

The distribution of phenotype among the 108 patients, including those of unknown genotype, was also consistent with previous studies. Probit analysis of the data showed the distribution to be bimodal with the antimode equaling 0.6. Thus 103 patients were classified as having the EM phenotype and 5 (4.6%) as having the PM phenotype. The calculated antimode was compatible with that published elsewhere [Bosso et al. 1996; Straka et al. 1995; Anthony et al. 1995] in that using those authors' antimode of 0.3 would have resulted in the same classifications.

However, two of the PM phenotypes were genotypically EM. Although only three mutant alleles were tested for, out of a possible 18 [Sachse et al. 1997], it would be highly unlikely for two subjects from the studied population to each carry a pair of the other 15. Moreover, subsequent tests of those patients (No. 6151, 7184) found them to have reverted to an EM phenotype. A third subject, of unknown genotype, also showed a phenotypic flip on the second testing. In this case, the movement was from an EM to a PM phenotype.

Medication was not a factor in the discrepant gene expression. Neither of the EM genotypes expressing a PM phenotype were on medication known to inhibit CYP2D6 activity. Such drugs were being taken by five of the genotyped patients all of whom had the EM phenotype and genotype. The fact that they were not phenotypically PM, despite taking inhibitory medication, was not surprising as only quinidine has been identified as an inhibitor strong enough to transform extensive CYP2D6 metabolizers into poor metabolizers, albeit transiently [Hou et al. 1996]. However, medication did appear to
suppress CYP2D6 activity as the patients on inhibitory drugs were clustered toward the right (slower) side of the EM distribution (Figure 2).

The "peak" at log([DM]/[DR]) = -1.2, seen in Figure 2, was only partly accounted for by coadministration of medication inhibitory to CYP2D6. For that reason it was decided to evaluate the data from the EM patients, excluding those on inhibitors, for the possibility of a secondary antimode. Probit analysis yielded one at log([DM]/[DR]) = -1.5, equivalent to a molar DM/DR ratio of 0.03.

The peak seen at the "slow" end of the EM population could be merely part of normal variation. However genotype, in terms of heterozygote vs. homozygote, would not appear to be a contributing factor (Table 2). Heterozygous and homozygous EM's were equally distributed on either side of the secondary antimode, 30% and 28% of the heterozygotes and homozygotes, respectively, being "slow" EM's. Funck-Brentano et al. [1992] showed a histogram of CYP2D6 activity among 110 healthy subjects which contained a small shoulder on the "slow" side of the EM peak, but there was no lack of concordance between geno- and phenotype nor were there any subjects within 0.5 logs of the left (EM side) of the antimode. In a study of psychiatric patients, Chen et al. have also shown an intermediate group of EM phenotypes. The distribution among 54 patients, phenotyped using DM and genotyped, appeared to be trimodal with a breakdown of 45:4:5 EM:"slow" EM:PM. There was no discrepancy between geno- and phenotype, but three of the four "slow" EM's had reported adverse events associated with the taking of various antidepressants compared with five of the 45 EM's.

In this study of a sick population, this phenomenon was exaggerated. There was an apparent peak at the slow end of the EM spectrum, two patients with CYP2D6 EM genotypes expressed a PM phenotype and another 4 patients with EM genotypes had [DM]/[DR] ratios greater than that of patient No. 1150 who was taking three CYP2D6 inhibitors (Figure 2, Table 2). Thus, there appears to be abnormal variation in CYP2D6 expression among this HIV-infected population.

Disease progression is one mechanism by which a phenotype can be altered. This has been observed for the enzyme NAT2 in two studies involving AIDS patients [Lee et al.
In the present study, no association was found between phenotype or pheno-subtype and a diagnosis of AIDS in the cross sectional study or with disease progression in the longitudinal study. The only disease-related factor which appeared to explain the observed "slow" EM peak was the presence of an active infection or tumour at the time of phenotype determination. The odds ratio associating a "slow" EM or PM phenotype with active disease was 4.6 (95% confidence interval 1.7 - 12.2; significant).

However, the longitudinal segment of the study failed to show any association between active disease and "slowness" even when the second phenotype data were analyzed cross sectionally.

There are two statistical factors which may mitigate against revealing significant effects within the longitudinal data. First there were only 40 subjects phenotyped more than once who were not taking CYP2D6 inhibitors. The second issue is one of noise. Within any individual, day to day variation in [DM]/[DR] ratio is expected. Ducharme (1996) found a relative range within healthy individuals tested up to six times within a year to be as high as 6.3-fold (i.e. the maximum [DM]/[DR] ratio was 6.3 times the minimum). To test statistical relationships between changes in pheno-subtype and changes in disease activity within individuals with any reasonable statistical power would require a lower noise level or a higher number of subjects.

These results implicate illnesses (acute rather than historic) as a significant factor in altering the expression of CYP2D6. However, the effect may be related to the underlying disease itself (i.e. HIV infection). The HI virus or HIV related fatty infiltration into the liver [Grunfeld et al. 1992] may have a degenerative effect on this organ's ability to metabolize drugs. This effect would not be detectable by standard liver function tests and would only manifest itself when the liver is challenged by a drug.

Such an HIV-mediated effect may in fact be secondary to changes in cytokine levels. TNF-α levels are increased in HIV-1 infected patients including children [Rautonen et al. 1991], drug users [Ayehunie et al. 1993], and Africans [Martin-Chollet et al. 1994]. Elevated TNF-α levels are detected in symptomatic AIDS patients during acute opportunistic infections [Lahdevirta et al. 1988]. Elevated IL-1 [Cox et al. 1990] and IL-6
[Breen et al. 1990] serum levels have been reported in HIV infections as well as the production of these cytokines by monocytic cells infected with HIV [Molina et al. 1990]. Although IL-1 levels can be higher in early disease and decline thereafter, IL-6 levels can be elevated in late disease and associated with elevated TNF-α, IgG and IgA levels [Mildvan et al. 1992]. It has been proposed that increased IL-6 production is associated with HIV disease progression [Breen et al. 1990].

IL-1 has been shown to reduce CYP activities in mice and rats [Ghezzi et al. 1986; Shedlofsky et al. 1987; Kurokohchi et al. 1992]. Administration of recombinant IL-1 or lipopolysaccharide (LPS)-induced inflammation, resulted in an in vitro reduction in the metabolic activities of CYP1A1, -2B1, -2C11, -2D1, -2E1 and -3A. The decrease in CYP2D1 activity in rat liver microsomes prepared from IL-1α treated animals was associated with a decrease in the level of CYP2D mRNA, suggesting that IL-1α down-regulated CYP2D activity at the mRNA level [Kurokohchi et al. ibid.]. Studies with IL-2 in rats have indicated that this cytokine increased the enzymatic activities of CYP1A1, -2B1, -2C11, -2D1 and -2E1 [Kurokohchi et al. 1993]. That study's results also indicated that IL-2 induced an increase in the amounts of immunoreactive CYP2D protein and its mRNA, suggesting that the enzymatic activities were up-regulated at the mRNA level.

In order to assess the effect of cytokines on drug metabolism in humans, LPS was administered to volunteers for one or two consecutive days [Shedlofsky et al. 1994]. The subjects were also administered a cocktail of antipyrine, hexobarbital and theophylline. The latter two drugs can be used as markers for CYP2C19 and CYP1A1 activities, respectively. After Day 1, no significant changes were observed in the metabolic clearance of the probe drugs. After Day 2, significant decreases in the clearance of all three drugs were observed. The decrease in antipyrine clearance correlated with initial peak levels of TNF-α and IL-6. The data demonstrate that in humans, the LPS induced inflammatory response decreased hepatic CYP-mediated drug metabolism and that TNF-α and IL-6 play a role in this response.

Thus, variation in CYP2D6 activity among HIV/AIDS patients could in part be due to the actions of cytokines such as IL-1, IL-6 or TNF-α, the serum levels of which are all
elevated at various times during HIV infection, particularly when opportunistic infections are active [Lahdevirta et al. 1988].

At the time of this study there was no reliable data with which to gauge the activity of HIV infection itself. Viral burden measurements were not widely available until late in the program so the effect of active illness on CYP2D6 expression, in terms of HIV infection, could not be addressed.

**Conclusion**

The treatment of HIV infection is rapidly moving towards polypharmacy, *i.e.* combination antiretroviral therapy, multiple prophylactic therapy, anxiolytics, etc. Each new combination carries the risk of metabolic drug interactions with increased toxicity and reduced efficacy. In this environment, knowledge of a patient's metabolic status will be important in designing an individual's treatment regimen. The potential of this approach has been illustrated in this work by the demonstration that changes in the expressed activity of CYP2D6 may occur within an HIV-positive population. These changes can include movement of EM's toward the PM phenotype and *EM* genotypes expressing the enzyme at a PM level.

The data showed the presence of active illness to be a factor in the inhibition seen in CYP2D6. While the mechanisms underlying the observed phenotypic changes have not been identified, they surely lie in a combination of drug interactions, disease progression, genotype and/or factors associated with the HIV infection itself such as cytokine action. These possibilities are currently under investigation.

These results also suggest that probe drug phenotyping with DM (an easy, safe, non-invasive probe of CYP2D6 enzyme activity) could be useful as a probe of general CYP metabolic activity in this patient population. Selection of tailored dosage regimens for individual patients should benefit from determination of the metabolic phenotype.
References


CHAPTER VI: General Conclusion

The influence of HIV infection and AIDS on the capacity of patients to metabolize medication was investigated using a systematic approach. Metabolic phenotypes and genotypes for two polymorphic drug metabolizing enzymes were established as were the capacities to glucuronidate and sulphate acetaminophen.

There were no factors uncovered which exerted changes in any pathway universally. Disease status (AIDS vs. not) and disease progression were found to play a role in depressing the expression of NAT2 whereas active disease seemed to dampen CYP2D6 expression. There was no relation between the two pathways. A few patients did show depressed activity for both pathways but they were not necessarily among the sickest (although some were). Conversely some very ill patients showed no perturbations in the expression of either pathway.

The pathways of glucuronidation and sulphation, probed as general processes, were found to be largely unaffected by any of the HIV-related factors investigated.

The major conclusions to be drawn from the research are:

1) Interindividual variation in drug metabolism within the HIV positive population is higher than among seronegative populations.

2) Prediction of metabolic capacity on the basis of genotype is not recommended in such a population.

3) Knowledge of the genotype is very useful so long as the phenotype is also known.

4) The phenotype can change over time within an individual.

5) The use of a take-home metabolic phenotyping kit is practical for determining the activity of multiple xenobiotic metabolizing pathways even in as complex a subject population as that examined here.

6) There is considerable scope for further research in this area. The presumption that cytokine activity may be central to the mechanisms which interfere with drug metabolism deserves consideration.
The author can make the following claims of originality:

1) The first systematic assessment of drug metabolism in an HIV infected population including the first longitudinal study.

2) The first study of multiple drug metabolizing pathways in a sick population using a take-home metabolic phenotyping kit.

3) The first study to elucidate the presumed discrepancy between NAT2 genotype and phenotype by determining both measures in the same population.

4) The first study to demonstrate conversion of genotypic CYP2D6 EM's to phenotypic PM's by processes unrelated to coadministered drugs.
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APPENDICES

Appendix 1: Protocol

Appendix 2: Informed consent form

Appendix 3: Patient questionnaire

Appendix 4: Case report form

DETERMINATION OF METABOLIC INDICES IN AIDS PATIENTS

1 AIMS AND OBJECTIVES

This study will explore the role of metabolic profile/phenotype in the response/toxicity ratio of medications commonly used in the management of AIDS. The aim of the research is to discover the key factors which follow or predict the metabolic changes in the AIDS wasting syndrome in order to guide physicians in the prescription of drugs to those patients and in the timing of nutritional intervention.

The study described herein aims to:

1) establish the association between individual patients' ability to metabolize drugs and the safety and effectiveness of those drugs;
2) monitor the metabolic capacity of AIDS patients;
3) determine changes in their ability to metabolize drugs as their disease progresses and their nutritional status wanes.

2 RATIONALE

Pronounced interindividual variations in response to usual doses of drugs have been recognized in clinical practice and many of those agents are activated or eliminated by metabolic routes that are under genetic control. The genetic control results in at least two distinct subgroups in the population which differ in their ability to perform certain biotransformations. Individuals that are deficient in their ability to metabolize certain drugs, relative to the mean rate and extent of metabolism, are called poor metabolizers or PM-phenotypes; extensive metabolizers, EM-phenotypes, have normal to fast metabolism. Which phenotype is affected by toxicity or lack of efficacy depends on the drug's mode of action. If, as in the case of encainide, the metabolite is the major active component, then a lack of efficacy is apparent in PM's. Alternatively, as with tricyclic antidepressants, the parent drug is the active component. Therefore, EM's may not respond due to the rapid rates of metabolism making it difficult to reach steady-state therapeutic ranges with normal doses.

The classification of patients as to metabolic polymorphism/capacity is possible through the use of innocuous probe drugs. These drugs are metabolized by well characterized hepatic microsomal enzymes and the level of those enzymes is reflected in the metabolite to parent ratio of the appropriate drug. The antitussive dextromethorphan (DM), for example, is metabolized in two steps by two isozymes.

A1.1
of the cytochrome P450 (CYP) family of drug-metabolizing enzymes. Thus the ratio of the O-desmethyl metabolite to its parent (DM) is an index of CYP2D6 activity while the N-desmethylDM/DM ratio is indicative of CYP3A4 activity. In equivalent manner, caffeine and acetaminophen, serve as probes for N-acetyltransferase and glucuronidation, respectively.

The frequency distribution of the levels of urinary parent/metabolite ratios is analyzed in the studied population. Probe drugs are chosen for the pathway (hepatic microsomal isozyme) of interest. EM's and PM's fall into two distinct groups, distributed below or above a certain level (determined by probit analysis of the data). Two of the most widely studied hepatic routes showing genetic polymorphism of drug metabolism are the debrisoquin/sparteine oxidation pathway (CYP2D6 probed with DM) and the N-acetylation pathway (probed with caffeine). Some of the drugs which co-segregate with the debrisoquin/sparteine metabolism are various β-blockers (metoprolol, propranolol), antiarrhythmics (encainide, perhexiline), antidepressants (desmethylimipramine, nortryptiline) and codeine. The adverse side-effects linked to this pathway are CNS toxicity, peripheral neuropathy, and excessive β-blockade. Codeine is metabolized to morphine via that pathway which may represent an important activation pathway. Drugs that are subject to N-acetylation polymorphism include sulfonamides (sulfamethazine), antidepressants (phenelzine), antiarrhythmics (procainamide), antihypertensives (hydralazine) and dapsone, which is also metabolized by CYP3A4. Some adverse therapeutic consequences of the acetylator phenotype are peripheral neuropathy and hepatitis. Recent evidence suggests that CYP3A4 may also be polymorphic.

Variations in drug metabolism occur within individuals depending on smoking, the intake of numerous compounds (e.g. drugs) known to inhibit or induce hepatic drug metabolizing enzymes or other factors. Nutritional status has also been shown to have an effect on drug metabolism. In malnourished subjects, drug metabolism can be reduced or increased, depending on the pathways involved.

Malnutrition is an almost inescapable aspect of AIDS which is complicated by metabolic abnormalities caused by the host response to HIV-infection. These abnormalities have been studied extensively in terms of protein and energy metabolism but the effect of malnutrition on drug metabolism in AIDS has not been studied nor has the molecular basis of the observed changes been investigated.

We are proposing to monitor in AIDS patients the functional levels of four metabolic pathways involved in the hepatic metabolism of drugs: 4-hydroxylation (CYP2D6), N-demethylation (CYP3A4), N-acetylation and glucuronidation. In our study, we plan to use probe drugs (DM, caffeine and acetaminophen) and to also monitor the metabolism of codeine (CYP2D6), dapsone (CYP3A4 and N-
acetyltransferase) and AZT (glucuronidation) in those patients so prescribed. Metabolic index values will be based on their metabolic ratios. The probes (dextromethorphan, caffeine and acetaminophen) are fairly innocuous and the sample collection is non-invasive (urine). The method of analysis will be by HPLC or capillary electrophoresis. It is expected that differences in the metabolism of the probes (e.g. DM for CYP2D6) will be paralleled by differences in substrate metabolism (e.g. codeine), that as caffeine metabolism increases (or decreases) so will that of dapsone. Indices of nutritional status, including body weight, serum albumin level and bioelectric impedance analysis, and liver function will also be assembled for each patient and multivariate analysis used to test correlations between drug-metabolic capacity and nutritional status.

The knowledge obtained as to the metabolic capacity of individuals and changes that may be effected during the course of their illness may be of great help in tailoring drug regimens which balance efficacy and toxicity and may provide an early indicator of the hepatic abnormalities which lead to cachexia.

3 STUDY DESIGN

3.1 Study Population

Sixty (60) patients will be asked to participate from the Immune Deficiency Treatment Centre of the Montreal General Hospital. All individuals will be asked to sign an informed consent prior to participation.

3.2 Inclusion Criteria

3.2.1 Asymptomatic Western Blot (or RIPA) confirmed HIV positive patients;
3.2.2 Age between 12 and 60 years old;
3.2.3 Mean CD4 count of 200 cells/mm³ or less, based on two (2) separate determinations, performed at intervals over no more than eight (8) weeks prior to the first experiment;
3.2.4 Patients must sign a written informed consent; patients under the age of 18 require written informed parental consent;
3.2.5 Negative pregnancy test for women of childbearing potential;
3.2.6 Body weight range of 80-120% the Life Insurance Underwriters Standard for age and height.

3.3 Exclusion Criteria

3.3.1 Active clinically significant medical problems. Patients with
haemophilia are permitted if considered clinically stable at the time of entry;

3.3.2 Liver abnormalities defined as SGOT/SGPT greater than 200 IU/L, or albumin less than 3.0 g/dL or prothrombin time > 15 seconds (except patients with haemophilia);

3.3.3 Renal dysfunction defined as: BUN > 10 mmol/L or creatinine > 100 µmol/L;

3.3.4 Haematologic abnormalities as defined by: haemoglobin < 120 g/L or WBC < 3,000/mm³ or platelet count < 70,000/mm³;

3.3.5 Chronic alcohol or drug abuse within 12 months prior to this study;

3.3.6 Women of child bearing potential, defined as a pre-menopausal female who is biologically capable of becoming pregnant; unless a negative pregnancy test is obtained and the patient agrees to use two (2) means of artificial birth control (condom plus an additional hormonal or mechanical method).

3.4 Dose

On the day of the test, the subject will first provide a pre-dose (blank) urine sample before being given one tablet containing 100 mg caffeine (NoDoz, Bristol Myers Squibb Co., Wakeups, Adrem Ltd. or Chase Caffeine Tablets, A.W. Chase) and one tablet containing 500 mg acetaminophen (Atasol Forte, Frank W. Horner Inc. or Tylenol Extra-Strength, McNeil Consumer Products Co.) with approximately 100 mL water and 10 mL of cough syrup containing 30 mg dextromethorphan (Robitussin-DM, A. H. Robins Co. Inc. or Balminil D.M., Rougier Inc.).

3.5 Sample Collecting and Handling

A 10 mL aliquot of the pre-dose (blank) urine sample will be retained frozen for analysis. At 4 h post-dose, the patients will be asked to collect a sample of their urine in the container provided. Three (3) 10 mL aliquots will be separated from the 4 hour specimen. The three aliquots will be retained in a freezer at -20°C until analyzed for phenotype/metabolic index.

4 POTENTIAL HAZARDS

All the administered medications are non-narcotic, non-prescription and available over the counter. A recognized but rare side effect of a single dose of NoDoz is agitation which is transient and the side effects of a single dose of Robitussin DM are rare and include nausea and dizziness.
Determination of Metabolic Index in AIDS Patients

Patient's Informed Consent Form

Dextromethorphan, Acetaminophen and Caffeine

The way in which your body responds to the medication(s) you are taking may be due to a specific and inherited pattern of metabolizing drugs. Further, the way in which you metabolize drugs may change as your disease state and/or nutritional status changes. In order to determine what pattern you have and how it might change, you will take:

- approximately two teaspoons (10 mL) of cough syrup containing the test drug dextromethorphan (DM) (Robitussin DM® or Balminil DM® - 30 mg/10 mL).
- a caffeine tablet (Wake-up®) or a cup of coffee
- and an extra-strength acetaminophen tablet (Tylenol® or Atasol®) with a glass of water (200 mL)

You can take the syrup and the tablets together, and this will be done on one occasion only but you may be asked to repeat the process on subsequent occasions.

On the day of the test you will abstain from coffee or any caffeinated beverage (colas, hot chocolate, tea).

You will collect two (2) spot urine samples (of about 20 mL, or four teaspoons, each): the first one before ingesting the syrup and tablets (pre-dose urine sample) and the second one 4 hours after taking the syrup and tablets (4-h urine sample). During those 4 hours, you need not collect your urine nor abstain from urinating. After 4 hours, you will be able to drink coffee or any caffeinated beverage.

Dextromethorphan is a non-narcotic, non-prescription drug commonly used to treat coughs. Robitussin DM® and Balminil DM® are cough syrups that are available over the counter. The uncommon side effects of a single dose of Robitussin®/Balminil DM® may include nausea and dizziness; but these effects are transient and disappear rapidly.

The Wake-up® tablet contains 100 mg of caffeine, which is approximately equivalent to a cup of regular coffee. A single dose of caffeine doesn’t usually induce side effects, but rarely, it may cause nausea, nervousness, insomnia, palpitations or headache; the possible side effects are transient and disappear rapidly.
The Tylenol® or Atasol® tablet contains 500 mg of acetaminophen, equivalent to less than two regular strength tablets. Acetaminophen is a common headache remedy available over the counter. Side effects from a single dose are exceedingly uncommon.

PCP Medications

Patients infected with the Human Immunodeficiency Virus (HIV) usually take dapsone (Avlosulfon) or Septra (sulphamethoxazole/trimethoprim) to treat or prevent pneumocystis carinii pneumonia (PCP). These patients may later require other medications such as cough suppressants, analgesics or antibiotics. Since these drugs’ use in treating PCP is relatively new, their interactions with other drugs in the human body is not completely known.

In order to understand how such medications are metabolized by your liver, you will take one or both of the following:

Ketoprofen

Two Apo-Keto tablets (100 mg ketoprofen) with a glass of water. Ketoprofen is a non-steroidal anti-inflammatory drug used in the management of pain. The uncommon side effects of a single dose of ketoprofen include stomach upset, but that would be transient and disappear rapidly. As in the test described above (cough syrup/caffeine), urine samples pre-dose and 4 hours post-dose will be required.

This test may be performed in conjunction with that described above (cough syrup/caffeine) with the ketoprofen replacing acetaminophen. Additional urine samples, therefore, would not be involved.

For certain subjects, depending on which medications are currently prescribed and other factors, the test will have to be performed in addition to the syrup/caffeine test, on a separate day.

Aminosalicylate Sodium

Septra is a very common medication in the management of HIV-related diseases (i.e. PCP) but many patients react badly to it. Adverse reactions to drugs, like rashes which sometimes affect people taking Septra, may in part be due to the way in which the drugs are metabolized. The component of Septra which most likely causes such adverse reactions is metabolized by an enzyme called NAT1. The medicine aminosalicylate sodium (Nemasol Sodium) which is sometimes prescribed for tuberculosis, is also metabolized by NAT1. The way in which you metabolize Nemasol should parallel the way you would metabolize Septra.
In order to determine the status of your NAT1 system, you will:

- provide 20 mL (about 4 teaspoons) of blood to test for NAT1 in your blood cells;
- take two 500 mg Nemasol Sodium tablets, about one tenth of a normal daily dose;
- collect two (2) urine samples, the first, of about 20 mL (4 teaspoons), before taking the pills (pre-dose urine sample) and the second one to consist of all your urine over the 2 hours after taking the pills (0-2 h urine sample). During those 2 hours, you need to collect all your urine.

You would take the Nemasol and provide the blood and urine on a different day from the one when you take the cough syrup.

Aminosalicylate sodium is used in conjunction with other drugs to treat tuberculosis. Side effects such as nausea, vomiting, abdominal pain or diarrhoea have been reported, but only in patients taking 20 - 24 tablets a day, the normal dosage. A single 2-tablet dose of Nemasol is very unlikely to cause any such effects. The tablets should be taken with a meal so as to minimize any gastrointestinal discomfort which might occur. Any side effects from a single dose would be transient and disappear rapidly.

Other Medications

As usual, when you visit the clinic, blood will be drawn. Should you be receiving other medications, some of that blood and some of the pre-dose urine you collect for this study may be measured for drug and metabolite concentrations.

Metabolic Genotype

Your pattern of metabolizing drugs can in part be due to what you inherited. In order that your pattern may be studied at the genetic level (the level of your DNA), you will provide a sample of 10 mL (about 2 teaspoons) of blood.

Data Collection and Confidentiality

You will fill out a short questionnaire designed to collect information which will be used to interpret the results of the study. The investigators might need access to your medical record, in order to gather additional relevant information. This questionnaire and a case report form containing all the collected information will be kept confidentially at the Montreal General Hospital.
No information by which you can be identified will be released or published. The results of this study may be published in medical journals or reported at medical research meetings. Individual patients will never be identified. At the end of the study, if you so wish you can be informed of the results.

Benefits

With this study, we aim to improve the management of AIDS by understanding how the way in which individual patients metabolize drugs might change with nutritional status and/or disease progression. Participating in this study may not give any direct benefit, but it may help physicians and scientists choose the best treatment for other patients suffering from AIDS and related diseases.

Voluntary Participation

Your participation in this study is totally voluntary. You are free not to participate or to withdraw your consent to participate in this study at any time, without any penalty or loss of medical care.

If the results of the present study warrant further investigation, you will be asked to participate by repeating the tests. If this occurs, you will be fully informed and your participation will be totally voluntary.

Written Consent

I have read all the above and the attached patient's information sheets. I asked questions and received answers on all matters I did not understand. I willingly give my consent to participate in this study. Upon signing, I will receive a copy of this consent form and of the information sheets.

I agree to participate.

PATIENT ___________________________________ DATE ________

INVESTIGATOR _______________________________ DATE ________

WITNESS ___________________________________ DATE ________
Appendix 3:
MONTREAL GENERAL HOSPITAL
Patient Questionnaire

Investigators: Drs. Wainer, Tsoukas and Falutz.

METABOLIC INDEX STUDY

Subject's name _________________________________
Medicare Number _______________________________
IDTC Treating Physician ___________________________

Date ____________________ (d/m/yr)

- When were you born __________________________ Age __________________ yrs
  (d/m/yr)

- How would you classify your ethnic origin?
  □ White          □ Black:          □ Asian
  □ Native American □ African-American □ Hispanic
  □ Arabic         □ African         □ West Indian
                    □ Other: __________________

- How tall are you? __________________

- When were you first diagnosed with HIV? __________ N/A □

- Are dapsone (Avlosulfon) or Septra (sulfamethoxazole/trimethoprim) currently prescribed for you? □ No
  □ Yes. please specify:
  
  Medication __________________________ Dose/frequency _______ Time of last pill

- Have either dapsone or Septra been prescribed for you in the past? □ No
- In the last 24 hours have you taken any of the following medications (except those taken for the test)?

<table>
<thead>
<tr>
<th>Medication</th>
<th>Dose/frequency</th>
<th>When you stopped (m/yr)</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

- Are you currently (within the last month) taking any over the counter medications, vitamins, other supplements, herbal extracts etc.? Please specify:

<table>
<thead>
<tr>
<th>Product</th>
<th>Amount</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>

- How many coffees or other caffeine containing beverages (e.g. tea, hot chocolate, cola) do you usually have per day?

  0  □
  1-4 □
  more than 5 □

- Before performing this test, when was your last cup of coffee (or tea/cola)?

  Day ______ Time ______

- How many alcoholic drinks do you have per week?

  0-7 □
  8-14 □
  more than 14 □
Do you smoke (tobacco)?
- no □
  - yes □
    - How long have you been smoking? ______ years
    - How many cigarettes per day? ______

Did you ever smoke?
- no □
  - yes □
    - When did you stop? __________
    - For how long (m/yr) did you smoke? ______ yrs
    - How many cigarettes per day? ______

Does anyone with whom you live smoke?
- no □
  - yes □
    - For how long, during your cohabitation, has he/she smoked? ______ years
    - How many cigarettes per day? ______

Do you use any other drugs?
- no □
  - yes □
    - Please specify drug(s), amount and frequency:
      __________________________
      __________________________
      __________________________
METABOLIC INDEX STUDY

IDTC Treating physician ________________________________

Patient's name ___________________________ Patient's study number __________

MGH chart number ______________________________ Medicare Number ______________________________

Date ____________ (d/m/y) Date of Birth ____________ (d/m/y)

- Weight ________ kg ________ lb
- Height ________ cm ________ in
- Body surface area ____ m² G ____ P ____ A ____
- Ethnic origin
  - [ ] White
  - [ ] Native American
  - [ ] Arabic
  - [ ] Black:
    - [ ] African-American
    - [ ] African
    - [ ] West Indian
  - [ ] Asian
  - [ ] Hispanic
  - [ ] Other __________________

Relevant medical history

Date of HIV diagnosis ____________ (d/m/y) N/A [ ]

CDC classification

- [ ] A1
- [ ] B1
- [ ] C1
- [ ] A2
- [ ] B2
- [ ] C2
- [ ] A3
- [ ] B3
- [ ] C3

CD4 _______(%) _______ (cells/mm³)

Significant abnormalities/ Major surgery no [ ] yes [ ]

A4.1
## General History

<table>
<thead>
<tr>
<th>No.</th>
<th>Condition</th>
<th>Date of onset (m/y)</th>
<th>Description</th>
<th>Date Ongoing resolved</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cardiac disease</td>
<td></td>
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<tr>
<td>2.</td>
<td>Hypertension</td>
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<tr>
<td>3.</td>
<td>Other vascular disease</td>
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<tr>
<td>4.</td>
<td>Diabetes</td>
<td></td>
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<tr>
<td>5.</td>
<td>Other metabolic endocrine</td>
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<td></td>
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<tr>
<td>6.</td>
<td>Hepatobiliary disease</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7.</td>
<td>GI disease</td>
<td></td>
<td></td>
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<tr>
<td>8.</td>
<td>Renal disease</td>
<td></td>
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<tr>
<td>9.</td>
<td>Other genito-urinary disease</td>
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<tr>
<td>10.</td>
<td>CNS disease</td>
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<tr>
<td>11.</td>
<td>Haematologic disease</td>
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<tr>
<td>12.</td>
<td>EENT disease</td>
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<td>13.</td>
<td>Pulmonary disease</td>
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<td>14.</td>
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<td>15.</td>
<td>Musculoskeletal disease</td>
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<tr>
<td>16.</td>
<td>Dermatologic disease</td>
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<tr>
<td>17.</td>
<td>Sexually transmitted diseases</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18.</td>
<td>Other (specify)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
II Surgery (specify)  

Year  
Reason  

________________________  
________________________  
________________________  

III Smoking  
☐ Never used  
☐ In past, but not presently  
☐ Current use (specify)  
☐ Cohabitation with a smoker  

IV Alcohol use  
☐ Never used  
☐ In past, but not presently  
☐ Light use (< 8 drinks/week)  
☐ Moderate use (8-14 drinks/week)  
☐ Heavy use (>14 drinks/week)  

V Drug abuse  
☐ None  
☐ In past, but not presently  
☐ Present use (specify)  

VI HIV-associated conditions  

<table>
<thead>
<tr>
<th>Condition</th>
<th>no</th>
<th>yes</th>
<th>Onset (m/y)</th>
<th>No. of episodes/recurrences</th>
<th>On-going</th>
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<td>☐</td>
<td>☐</td>
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<tr>
<td>Oral candida</td>
<td>☐</td>
<td>☐</td>
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<tr>
<td>Oesophageal candidiasis</td>
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<tr>
<td>HSV</td>
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<td>☐</td>
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<td>☐</td>
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<tr>
<td>Herpes zoster</td>
<td>☐</td>
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</table>
### VII Medication History

**Allergies**

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<th>Drug</th>
<th>Brief description of reaction</th>
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</table>

**Prior PCP prophylaxis therapy**

<table>
<thead>
<tr>
<th>Drug:</th>
<th>Dose/Frequency:</th>
<th>Start date:</th>
<th>End Date:</th>
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</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>
Current prophylaxis  
   no □  
   yes □

Drug: __________________ Brand name: __________________ Start date: ______

Current medications:  
   no □  
   yes □ Specify:

<table>
<thead>
<tr>
<th>Medication</th>
<th>Indication</th>
<th>From (m/v)</th>
<th>To (m/v)</th>
<th>Dose/Frequency</th>
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</table>

Investigational Drugs:  
   no □ yes □

Specify: ______________________________________________________

Non-prescription medications/supplements  
   no □ yes □

Specify: ______________________________________________________
Appendix 5:

A Validated Method for the Determination of Paracetamol and its Glucuronide and Sulfate Metabolites in the Urine of HIV+/AIDS Patients Using Wavelength-Switching UV Detection

A. Di Girolamo,\textsuperscript{2a} W.M. O'Neil\textsuperscript{b} and I.W. Wainer\textsuperscript{d}

\textsuperscript{a}Département de Pharmacologie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada; \textsuperscript{b}Abbott Laboratories Limited, St.Laurent, Quebec, Canada; \textsuperscript{c}Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, Quebec, Canada; \textsuperscript{d}Pharmacokinetics Division, Montreal General Hospital, Montreal, Quebec, Canada

\textsuperscript{1}This manuscript is dedicated to Professor Anthony Fell in recognition of his contributions to the pharmaceutical and biomedical applications of multiple wavelength and diode array techniques.
Abstract:

Paracetamol is a safe drug which has been used as an in-vivo probe to determine phase II metabolism in a HIV+/AIDS population. Due to the biohazard nature of HIV-infected samples, a high-performance liquid chromatography (HPLC) assay which offers minimal sample manipulation and maximal specificity was developed. This reverse-phase HPLC method uses wavelength-switching UV detection for the simultaneous determination of paracetamol and its glucuronide and sulfate metabolites in HIV-infected urine samples. The solvent system involves a simple isocratic elution with a composition of 50mM sodium acetate buffer, pH adjusted to 3.5: acetonitrile [96:4 v/v] modified with 0.35% trifluoroacetic acid. The validated method is highly reproducible with an inter-assay variation of less than 7%. The method also shows good precision and sensitivity, making it an ideal assay for phenotyping studies to determine the extent of glucuronidation and sulfation activities.
1. Introduction

The intra-individual pharmacokinetics of many drugs vary considerably. These differences are largely due to differences in the metabolism, distribution and elimination of the therapeutic agents [1]. Metabolic differences are often the result of genetic polymorphism, drug interactions or environmental factors such as disease status, diet, lifestyle, etc. For enzymes which are genetically polymorphic, their activity falls into two clearly defined categories: individuals with a reduced capability to metabolize certain drugs i.e. "poor metabolizers" and those who demonstrate a regular pattern of metabolism i.e. "extensive metabolizers".

While genetic polymorphism is responsible for many inter-individual differences in response to drug therapies, much of the variability is due to non-genetically produced variations in the expression of these drug-metabolizing enzymes [2].

Changes in the relative levels and activities of metabolizing enzymes can be produced by drug interactions, environmental factors (i.e. smoking and alcohol intake), and clinical status such as disease progression [3] or malnutrition [4]. This implies that the expression of metabolic activity of a certain enzyme may change as a result of degeneration of a person's health. This phenomenon has been demonstrated in AIDS patients for the non-microsomal enzyme N-acetyltransferase II (NAT2) [5-7] and for the microsomal enzyme CYP2D6 [8], where patients with "extensive metabolizer" genotypes expressed "poor metabolizer" phenotypes as a result of disease status.

The genetic basis for polymorphic metabolism has been identified for a number of enzymes such as CYP2D6 and NAT2 [7,8]. The functional activities of these enzymes as well as many others can be determined by using probe drugs such as caffeine (NAT2) or dextromethorphan (CYP2D6) [7,8]. In critically ill patients, probe-drug phenotyping can be a key element in designing clinical treatment. Paracetamol (APAP) is one such probe drug.

APAP is a widely used and relatively safe analgesic/antipyretic. When given in therapeutic doses it is metabolized primarily by conjugation to form glucuronide and sulfate derivatives (phase II metabolism) [9-12], Figure 1. A small proportion is oxidized by the cytochrome P450 system to produce a highly reactive intermediate metabolite N-acetyl-P-benzoquinoneimine (NABQI) which is usually detoxified by conjugation with...
glutathione [9,13]. When glutathione stores are depleted, the formation of NABQI can result in liver injury [12,14].

APAP is an ideal drug for the study of factors influencing phase II drug metabolism in humans [15]. It is a safe, user-friendly probe-drug which can be used to determine the phase II metabolism in HIV+/AIDS patients. The extent of glucuronidation is important in antiretroviral therapy since it is the primary pathway responsible for the metabolism of AZT, and, to a lesser extent, is involved in the metabolism of protease inhibitors. As treatment for HIV moves towards combination therapy of nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs), AZT remains the cornerstone of this combination therapy. The addition of one or more PIs to the treatment regimen is quickly becoming the standard of care in HIV. Therefore, phase II metabolism plays an important role in the pharmacological and toxicological outcomes of these therapeutic agents in the HIV+/AIDS population.

Many high performance liquid chromatography (HPLC) methods exist for the assay of APAP and its metabolites in biological fluids [16-21]. However, many of these assays were developed for toxicological purposes and used for an otherwise healthy population. These assays did not suit the requirements for more detailed studies of phase II metabolism in a very ill population exposed to polypharmacy. Our present studies involve the determination of APAP and its glucuronide and sulfate metabolites in multiple, HIV-infected urine samples. Due to the biohazard nature and the multiple component matrix of the samples, the method required minimum sample manipulation and maximum specificity and sensitivity. This paper reports the development of a specific HPLC assay using wavelength-switching UV detection for the simultaneous determination of paracetamol and its glucuronide and sulfate metabolites in urine samples of HIV-infected patients. Results of validation studies including recovery, accuracy and precision, and sensitivity of this assay are reported.

2. Experimental

2.1. Chemicals

4-acetamidophenol (paracetamol) and β-acetamidophenol-β-d-glucuronide (paracetamol-glucuronide) were purchased from Sigma Chemical Company (St. Louis,
MO, USA). Paracetamol-sulfate was a gift from MacNeil Consumer Products (Fort Washington, PA, USA). HPLC-grade acetonitrile and glacial acetic acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Standard stock solutions

Aqueous stock solutions of paracetamol (APAP), paracetamol-glucuronide (APAP-G) and paracetamol-sulfate (APAP-S) were each prepared in polypropylene tubes at concentrations of 0.21 mg/ml, 8.45 mg/ml and 5.00 mg/ml respectively and stored at -20°C.

2.3. Separation Chromatography

2.3.1 HPLC System

The reverse-phase chromatographic system was composed of a Spectra Physics binary pump model P1500 and a Spectra Physics SP8875 autosampler equipped with a 20-ml sample loop, and a Spectra Physics UV1000 detector (Spectra-Physics, San Jose, CA, USA). A Spectra Physics Datajet integrator connected to a Spectra 386 computer using the software Winner on Windows was used for electronic data collection. Chromatographic separation of paracetamol and its metabolites was carried out with a Phenomenex ODS column 25cm X 4.6mm I.D. (Phenomenex Company, Torrance, CA, USA) fitted with a 5 micron C18 guard column (Regis Chemical Company, Morton Grove, IL, USA).

2.3.2 Mobile Phase

The compounds of interest were separated with a mobile phase composed of sodium acetate buffer [50 mM, pH 3.5]: acetonitrile [96:4 v/v] modified with 0.35% TFA. At 25 minutes, the acetonitrile composition was increased to 25%; at 30 minutes the mobile phase was returned to the original composition and the column was re-equilibrated for 15 minutes.

2.3.3 UV Detection

Detection of compounds was by UV, using a wavelength-switching program. The UV detector was programmed at 260 nm from 0 to 14 minutes; at 14 minutes the UV detector switched to 240 nm and autozeroed, and continued to detect at this wavelength until 25 minutes. At 25 minutes, the UV detector switched back to 260 nm. The detector

A5.5
was autozeroed at the beginning of each injection.

2.3.4 Chromatographic conditions

The chromatography was carried out using a flow rate of 1.0 ml/min at ambient temperature.

2.4 Probe-Drug Phenotyping Method

One hundred and fifty (150) subjects actively followed at the Montreal General Hospital (MGH) Immunodeficiency Treatment Centre (IDTC) and 36 seronegative controls were recruited into this study. IRB approval from the MGH Ethics Committee had been obtained prior to the start of the study. Written informed consent was obtained from each subject prior to the initiation of the phenotyping studies. Clinical data including a detailed HIV history, concomitant medications, past illnesses and current clinical status was obtained at time of enrollment. The participants were also given a questionnaire to complete as part of the phenotyping protocol.

Participants in this study were probe-drug phenotyped with a single oral dose of 500 mg of paracetamol (Atasol Forte®). A kit containing a 500 mg tablet of Atasol Forte®, and two urine specimen containers properly labeled were given to the participants along with instructions for dosing and storing of samples. Prior to the ingestion of the probe-drug, a pre-dose blank urine (20-40 ml) sample was obtained. Following the ingestion of the paracetamol, a 4-hour spot urine (20-40 ml) was obtained. The urine samples were returned to the Pharmacokinetics/Pharmacogenetics laboratory at the Montreal General Hospital. The samples were pipetted into four polypyrene tubes (4 ml), each containing an aliquot of 3 ml of urine. The samples were heated at 60 °C for 60 minutes providing for the inactivation of any virus and then stored at -20 °C until analysis.

2.4.1 Sample preparation

After thawing, the urine samples were diluted 1:20 with demineralized water to give a final volume of 1 ml. One hundred (100) µl of the sample was transferred to a 200-µl polypropylene autosampler vial and 20 µl was injected onto the HPLC column.

2.5. Standard curves

A 6-point standard curve was prepared by adding known concentrations of paracetamol and its metabolites, covering the ranges anticipated in the study to drug-
free pooled and filtered urine. A 5 ml stock solution of spiked urine diluted 1:20 containing APAP: 50 μg/ml, APAP-G: 5000 μg/ml and APAP-S: 2000 μg/ml was prepared. This stock solution was serially diluted to obtain concentrations ranging from 5-50 μg/ml of APAP: 500-5000 μg/ml of APAP-G and 200-2000 μg/ml of APAP-S.

2.6 Assay validation

For intra-day and inter-day validation studies, control samples were prepared from drug-free pooled and filtered urine spiked with paracetamol and its metabolites added at three different levels. The highest level of concentration contained 40.0 μg/ml of APAP, 4000.0 μg/ml of APAP-G and 1600.0 μg/ml of APAP-S. The medium level of concentration contained 30.0 μg/ml of APAP, 3000.0 μg/ml of APAP-G and 1200.0 μg/ml of APAP-S. The lowest level of concentration contained 5.5 μg/ml of APAP, 550.0 μg/ml of APAP-G and 220.0 μg/ml of APAP-S.

Recovery of the compounds of interest was tested in urine at the three different levels mentioned above. The control samples used in the validation were stored at -20°C and have been re-assayed over a six-month period. There were no significant changes in the chromatographic results indicating that the samples are stable for at least this period of time.

3. Results and discussion

3.1 Chromatography

The paracetamol-glucuronide and paracetamol-sulfate were detected at 260 nm with retention times of 4.8 and 8.5 minutes respectively. Paracetamol was detected at 240 nm with a retention time of 19 minutes. Under the chromatographic conditions utilized in this study, baseline separation of paracetamol and its metabolites was accomplished in biological samples.

The detection of APAP-G and APAP-S at a wavelength of 260 nm allows for the elimination of background noise with minimal loss of sensitivity, since both these compounds are present in large concentrations. Switching to 240 nm to detect the APAP peak increases the sensitivity and allows for maximal UV absorbance of this peak which has the smallest urinary concentration and is present in only 1/100 of the concentration.
of the other two peaks.

No interfering peaks were found in the pre-dose blank urine. Trace A in Fig. 2 is a chromatogram of a pre-dose blank urine, trace B is a chromatogram of a spiked urine; and trace C is a chromatogram of a patient sample, 4 hours post-dosing with 500 mg of APAP.

3.2 Validation

Recoveries of the compounds of interest were calculated at three levels between the limits of quantification by comparing the peak areas of the concentrations from spiked urine samples with peak areas from spiked aqueous samples. The recoveries were equal to or greater than 100% for all compounds with a C.V. (coefficient of variation) of less than 6%. The results are tabulated in Table 1.

A linear correlation-response was found over a range of 5 - 50 µg/ml for APAP, 500-5000 µg/ml for APAP-G, and 200-2000 µg/ml for APAP-S. The correlation coefficients (r) were in the range of 0.9986 to 0.9999 while the regression coefficients (r²) ranged from 0.9971 to 0.9997.

The results of the intra- and inter-day precision and accuracy are given in Table 2. In all cases, accuracy was within 13% of the theoretical for both intra- and inter-day variability. The precision calculated as the coefficient of variation (C.V.) was less than 5% for intra-day variation and less than 7% for inter-day variation for all compounds.

4. Conclusion

The validated method described is a relatively simple, sensitive and rapid assay which can be used in phenotyping studies to determine in-vivo, phase II metabolism. It is currently in use in a study of disease and drug effect on phase II metabolism in HIV+/AIDS patients. Urine samples from 150 HIV+/AIDS patients and 36 seronegative controls have been analyzed using this method. Results from this study will be reported elsewhere.
Acknowledgements

This work was supported in part by a grant to Professor Irving W. Wainer from the Canadian Foundation for AIDS Research (CANFAR). The invaluable help of Karen Fried is also gratefully acknowledged.

REFERENCES:


Table 1
Urinary recovery of APAP and its metabolites in human urine samples (n=5)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Recovery (%)</th>
<th>C.V. (%)</th>
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</thead>
<tbody>
<tr>
<td>APAP</td>
<td>5.5 µg/ml</td>
<td>103.9</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>30.0 µg/ml</td>
<td>108.2</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>40.0 µg/ml</td>
<td>119.3</td>
<td>5.9</td>
</tr>
<tr>
<td>APAP-G</td>
<td>550.0 µg/ml</td>
<td>103.4</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>3000.0 µg/ml</td>
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<td></td>
<td>4000.0 µg/ml</td>
<td>103.6</td>
<td>1.3</td>
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<tr>
<td>APAP-S</td>
<td>220.0 µg/ml</td>
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<td>4.7</td>
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<td></td>
<td>1200.0 µg/ml</td>
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<td></td>
<td>1600.0 µg/ml</td>
<td>101.9</td>
<td>2.0</td>
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Table 2
Accuracy and Precision of the Analysis of APAP and its metabolites in human urine samples

**Intra-day validation (n=3)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Actual conc.</th>
<th>Measured conc. (mean ± S.D.)</th>
<th>Accuracy (%)</th>
<th>C.V. (%)</th>
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<tbody>
<tr>
<td>APAP</td>
<td>5.5 µg/ml</td>
<td>6.2 ± 0.094</td>
<td>113.1</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>30.0 µg/ml</td>
<td>31.4 ± 1.056</td>
<td>104.7</td>
<td>3.36</td>
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<tr>
<td></td>
<td>40.0 µg/ml</td>
<td>42.9 ± 0.659</td>
<td>106.4</td>
<td>2.17</td>
</tr>
<tr>
<td>APAP-G</td>
<td>550.0 µg/ml</td>
<td>600.8 ± 25.859</td>
<td>109.2</td>
<td>4.30</td>
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<tr>
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<td>3000.0 µg/ml</td>
<td>2943.6 ± 45.325</td>
<td>98.1</td>
<td>1.54</td>
</tr>
<tr>
<td></td>
<td>4000.0 µg/ml</td>
<td>4036.6 ± 58.479</td>
<td>100.9</td>
<td>1.44</td>
</tr>
<tr>
<td>APAP-S</td>
<td>220.0 µg/ml</td>
<td>249.2 ± 7.328</td>
<td>113.3</td>
<td>2.94</td>
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<tr>
<td></td>
<td>1200.0 µg/ml</td>
<td>1235.2 ± 18.807</td>
<td>102.9</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>1600.0 µg/ml</td>
<td>1654.4 ± 23.713</td>
<td>103.4</td>
<td>1.43</td>
</tr>
</tbody>
</table>

**Inter-day validation (n=3 per day x 4 days)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Actual conc.</th>
<th>Measured conc. (mean ± S.D.)</th>
<th>Accuracy (%)</th>
<th>C.V. (%)</th>
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</thead>
<tbody>
<tr>
<td>APAP</td>
<td>5.5 µg/ml</td>
<td>6.2 ± 0.169</td>
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<tr>
<td></td>
<td>30.0 µg/ml</td>
<td>31.9 ± 1.250</td>
<td>106.4</td>
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<tr>
<td></td>
<td>40.0 µg/ml</td>
<td>43.6 ± 1.222</td>
<td>108.3</td>
<td>2.19</td>
</tr>
<tr>
<td>APAP-G</td>
<td>550.0 µg/ml</td>
<td>604.1 ± 37.896</td>
<td>109.8</td>
<td>6.27</td>
</tr>
<tr>
<td></td>
<td>3000.0 µg/ml</td>
<td>3033.0 ± 104.535</td>
<td>101.1</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>4000.0 µg/ml</td>
<td>4112.3 ± 98.216</td>
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<tr>
<td>APAP-S</td>
<td>220.0 µg/ml</td>
<td>254.0 ± 8.633</td>
<td>115.4</td>
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<tr>
<td></td>
<td>1200.0 µg/ml</td>
<td>1263.7 ± 40.085</td>
<td>105.3</td>
<td>3.2</td>
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<tr>
<td></td>
<td>1600.0 µg/ml</td>
<td>1676.1 ± 39.730</td>
<td>104.3</td>
<td>2.0</td>
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</tbody>
</table>
Figure 1: Paracetamol and its primary metabolites: paracetamol-glucuronide and paracetamol-sulfate (conjugation of -OH group).
Figure 2: A) Chromatogram of blank urine. B) Chromatogram of drug-free urine spiked with 40 µg/ml of APAP, 4000 µg/ml of APAP-G and 1600 µg/ml of APAP-S. C) Chromatogram from a sample of a patient, 4 hours post-dosing with APAP.